ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 32 FASC. 2—3

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Stockholm 1954 · P. A. Norstedt & Söner

30/11 1954

Redigenda curavit

PROFESSOR G. LILJESTRAND

KAROLINSKA INSTITUTET

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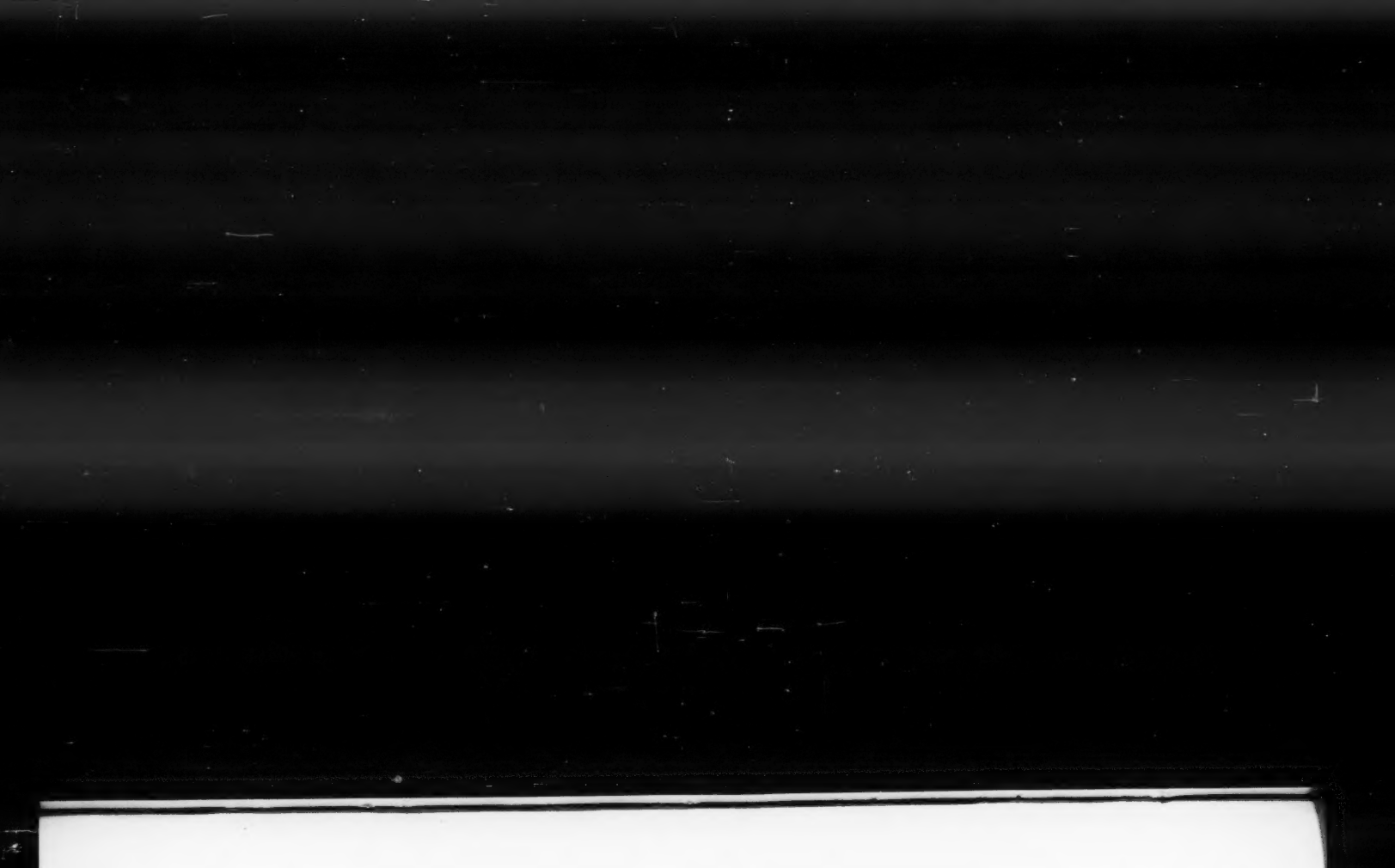


Department of Physiological Chemistry, University of Lund, Sweden

The Intestinal Absorption of Linoleic-1-^{14}C acid.

By

ROLF BLOMSTRAND.

Received 20 March 1954.

The intestinal absorption of a labelled monoethenoid acid, oleic-1-^{14}C acid, has recently been investigated in the rat by BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954). The major part of the absorbed labelled acid was recovered in the neutral fat of the lymph to the same extent as palmitic acid regardless of whether it was fed as free acid or as triolein.

Regarding the intestinal absorption of the diethenoid fatty acids very little is known. REISER and BRYSON (1951) working with conjugated linoleic acid recovered the labelled acid in the lymph fat of the rat regardless of whether the acid was fed as free acid or as glyceride. The recovery, however, was less than that obtained by us using labelled oleic acid.

In order to throw more light on the absorption of the unsaturated fatty acids, we have in the present study fed linoleic-1-^{14}C acid to rats with a thoracic duct fistula. To find out if there is any difference between saturated and unsaturated long chain fatty acids in the absorption and recovery in the lymph fat, a comparison has been made between labelled palmitic, oleic and linoleic acid.

Experimental.

Linoleic-1-^{14}C acid was prepared by BERGSTRÖM, PÄÄBO and ROTTENBERG. The material used in this investigation contained about 10 per cent conjugated acid.

The rats were given three different fat mixtures containing labelled linoleic acid (table 1).

A. Methyl linoleate-1-^{14}C.
B. 20 per cent methyl linoleate-1-^{14}C dissolved in olive oil.
C. A mixture of the fatty acids obtained from mixture B after saponification and extraction. The specific activities of the fatty acids from the mixtures in A—C were about 1,500 c.p.m. per mg.

In order to compare the absorption and recovery in the lymph of unsaturated and saturated fatty acids under identical conditions, olive oil containing 0.2 per cent palmitic-1-^{14}C acid was given to 5 rats (table 2).

The animals were treated as earlier described by BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954). On the first post-operative day the rats were fed by stomach tube 0.5 ml of a fat mixture while under light ether anesthesia. The thoracic lymph was then collected for 24 hours, after which time the animals were killed and the intestinal tract removed in order to determine how much of the activity that was unabsorbed. The expired carbon dioxide was collected from some animals.

The chemical methods of analyses and isotopic analyses used were essentially the same as described earlier (BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954)). The total fat from the lymph samples was separated into neutral fat and phospholipids on columns of silicic acid, and saponified according to BORGSTRÖM (1952).

In table 3 the present data are compared with those obtained by BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954) using labelled oleic acid.

Results.

The lymph experiments using different fat mixtures of labelled linoleic acid are shown in table 1. No apparent differences in the absorption values of the different fat mixtures were found. They all reached a maximum absorption of about 95 per cent with means of 85.1, 90.3 and 83.5 per cent respectively.

The mean values for the recovered activity in the lymph were 66.0, 58.2 and 57.2 per cent of the absorbed activity. About 4 per cent of the activity in the lymph fat was found in the phospholipid fatty acids.

In figure 1 the rate of appearance of the activity in the expired carbon dioxide is seen after feeding labelled methyl linoleate or palmitic-1-^{14}C acid in olive oil to rats having a thoracic duct fistula. After feeding labelled methyl linoleate, 4.8 and 6 per cent of the absorbed activity was found in the expired carbon dioxide. Corresponding figures after feeding labelled free palmitic acid in olive oil were 2.2 and 3.7 per cent.

The lymph experiments using palmitic-1-^{14}C acid dissolved in olive oil are summarized in table 2.

Table 1.

Recovery of activity in lymph fatty acids after oral administration of linoleic-1-^{14}C acid to rats.

Rat No.	Administration form	Per cent of administered activity absorbed	Per cent of absorbed activity recovered in lymphs fatty acids	Per cent of lymph fat activity found in	
				glyceride fatty acids	phospholipid fatty acids
1	A	92.3	88.0	97.3	2.7
2		64.1	40.6	96.1	3.9
3		89.0	71.6	—	—
4		95.0	63.9	—	—
		M. 85.1	M. 66.0		
5	B	97.3	63.8	97.1	2.9
6		90.6	59.0	96.2	3.8
7		96.7	76.5	95.9	4.1
8		68.8	41.3	93.8	6.2
9		98.1	50.6	—	—
		M. 90.3	M. 58.2		
10	C	95.8	51.4	95.4	4.6
11		54.8	48.0	95.6	4.4
12		87.6	61.9	94.7	5.3
13		95.9	67.4	96.3	3.7
		M. 83.5	M. 57.2		

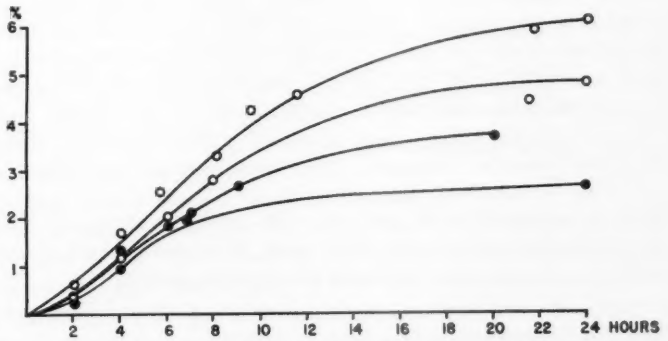


Fig. 1. Percentage of absorbed activity in expired carbon dioxide after feeding labelled methyl linoleate ○—○ and labelled palmitic acid ●—● to rats with a thoracic duct fistula.

In table 3 the mean and the extremes for the absorption and recovery in lymph fat of the different fat mixtures are given.

Table 2.

Recovery of activity in lymph fatty acids after oral administration of 0.2 per cent palmitic-1-^{14}C acid dissolved in olive oil.

Rat No.	Per cent of administered activity absorbed	Per cent of absorbed activity recovered in lymph fatty acids	Per cent of lymph fat activity found in:	
			glyceride fatty acids	phospholipid fatty acids
1	97.5	58.5	97.5	2.5
2	91.2	73.9	98.1	1.9
3	94.1	65.5	97.6	2.4
4	95.1	75.0	96.0	4.0
5	89.6	64.0	96.5	3.5
	M. 93.5	M. 67.4		

Table 3.

Recovery of different labelled fatty acids in lymph lipids in per cent of activity absorbed after oral administration.

Number of rats	Fat administered	Per cent of fed activity absorbed	Per cent of absorbed activity recovered in lymph lipids
5	Palmitic-1-^{14}C acid in olive oil	93.5 89.6—97.5	67.4 58.5—75.0
5	Oleic-1-^{14}C acid	78.2 65.5—91.8	63.0 38.1—96.4
4	Triolein labelled in the acid part	76.2 56.5—92.1	65.5 50.5—82.9
4	Linoleic-1-^{14}C acid in a mixture of fatty acids from olive oil	83.5 54.8—95.8	57.2 48.0—67.4
5	Methyl linoleate-1-^{14}C	85.1 64.1—95.0	66.0 40.6—88.0
4	Methyl linoleate-1-^{14}C in olive oil	90.3 68.8—98.1	58.2 41.3—76.5

Also, a saturated acid, palmitic-1-^{14}C acid, is compared with the unsaturated oleic and linoleic acids. It is seen that no greater differences between the different fat mixtures exist.

Discussion.

It has been shown in this investigation that the larger part of absorbed linoleic acid can be recovered in the lymph from the thoracic duct. The finding that linoleic acid enters the lymphatic pathway after absorption is thus in accord with the investigations

of REISER and BRYSON (1951) and REISER, BRYSON, CARR and KUIKEN (1952) who used conjugated linoleic acid. The percentages of the absorbed activity recovered in lymph were not influenced by the form in which the labelled fatty acid was administered *i. e.* as methyl ester or as free acid.

About 4 per cent of the activity of the lymph fat was found in the phospholipid fatty acids. This is almost the same figure as BLOOM, CHAIKOFF, REINHARDT, ENTENMAN and DAUBEN (1950) and BORGSTRÖM (1951) found for palmitic acid and also found in this investigation (table 2).

Rats having a thoracic duct fistula and fed methyl linoleate expired about the same amount of the absorbed activity as was expired by rats in previous experiments using oleic acid (BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954)). When labelled palmitic acid was given the absorbed activity in the expired carbon dioxide was somewhat lower but the difference is not significant.

It must be remembered here that the fed material contained about 10 per cent conjugated linoleic acid. It is possible that there is a difference in the rate of oxidation of conjugated and nonconjugated linoleic acid. This remains, however, to be investigated.

The possibility that the activity may reach the systemic circulation via the portal system, by way of lymphatic venous anastomoses or after degradation of the labelled fatty acid during its passage through the intestinal mucosa has been pointed out earlier by BLOOM, CHAIKOFF, REINHARDT, ENTENMAN and DAUBEN (1950) and by BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954). It is interesting to note in this connection that in another investigation (BLOMSTRAND 1954) decanoic-1-^{14}C acid was given to rats with a thoracic duct fistula and that here about 20 per cent of the absorbed activity was recovered in the expired carbon dioxide during the following six hours. Furthermore, BLOOM, CHAIKOFF and REINHARDT (1951) and KIYASU, BLOOM and CHAIKOFF (1952) have shown that decanoic acid is absorbed mainly via the portal system. Perhaps the most plausible explanation for the present findings is that the longer saturated and unsaturated fatty acids can be absorbed to a slight extent via the portal pathway. However, it must also be considered that fat absorption is presumably not entirely normal in cannulated animals.

A comparison of the amount of activity absorbed and the amount of activity recovered in the lymph fat between the saturated palmitic-1-^{14}C acid and the unsaturated oleic-1-^{14}C and linoleic-1-^{14}C acids showed no apparent differences.

From the findings in this investigation it can be concluded that there are no differences in the route of absorption of saturated and unsaturated higher fatty acids.

In humans similar results have been obtained by FERNANDES (1953) after feeding saturated and unsaturated fatty acids to a child whith chylothorax.

Summary.

1. A study on the transport of ^{14}C labelled linoleic acid in the rat via the thoracic duct has been made.

2. The major part of the absorbed labelled linoleic acid was recovered in the lymph neutral fat regardless of the form in which it was fed.

3. Some of the absorbed activity was recovered in the expired carbon dioxide and possible explanations for this fact are discussed.

4. A comparison has been made of the absorption of palmitic, oleic and linoleic acid and it is concluded that there are no differences in the route of absorption of saturated and unsaturated long chain fatty acids.

The author is grateful for the labelled linoleic acid placed at my disposition by Professor BERGSTRÖM.

This work has been supported by a grant from the Medical Faculty of the University of Lund, and "Knut och Alice Wallenbergs Stiftelse".

The technical assistance of Mrs. A. M. Andersson is gratefully acknowledged.

References.

BERGSTRÖM, S., R. BLOMSTRAND and B. BORGSTRÖM, Biochem. J. 1954. In press.

— K. PÄÄBO and M. ROTTENBERG. To be published.

BLOMSTRAND, R., To be published.

BLOOM, B., I. L. CHAIKOFF and W. O. REINHARDT, Am. J. Physiol. 1951. *166*. 451.
— — —, C. ENTENMAN and W. G. DAUBEN, J. Biol. Chem. 1950. *184*. 1.
BORGSTRÖM, B., Acta Chem. Scand. 1951. *5*. 643.
— Acta Physiol. Scand. 1952. *25*. 101.
FERNANDES, J., Fatabsorption studied in a child with chylothorax. Thesis. Leiden 1953.
KIYASU, J. Y., B. BLOOM and I. L. CHAIKOFF, J. Biol. Chem. 1952. *199*. 415.
REISER, R. and M. J. BRYSON, Ibidem 1951. *189*. 87.
REISER, R., M. J. BRYSON, M. J. CARR and K. A. KUIKEN, Ibidem 1952. *194*. 131.

From the Institute of Medical Physiology, University of Copenhagen.

Studies on the Lung Diffusion Coefficient for Carbon Monoxide in Normal Human Subjects by means of $C^{14}O$.

By

POUL KRUHØFFER.

Received 9 April 1954.

Introduction.

Arterial hypoxaemia during muscular exercise or even during rest is one of the most prominent signs of pulmonary insufficiency. Among the factors which may be responsible for the appearance of hypoxaemia are such changes in the area and thickness of the alveolar membrane, which lead to a reduction in its capacity for passage of gases by diffusion. A complete analysis of the functional state of the lungs must therefore include a determination of the overall "diffusing capacity" of the alveolar membrane.

The diffusing capacity of any membrane, simple or complex, may be expressed by a membrane diffusion coefficient, *i. e.* the amount of a gas which diffuses through the membrane in unit of time if unit of gas tension difference is maintained everywhere across the membrane. For the whole alveolar membrane, the term *lung diffusion coefficient* (or *constant*) has been applied, and in conventional units is defined as the number of ml (0°, 760 mm Hg) of the gas in question which would diffuse across the total alveolar membrane per minute if 1 mm Hg tension difference of this gas existed everywhere across it.

From this definition it is evident that only for such gases where a known tension difference can be established uniformly across the whole alveolar membrane, does the determination of the lung

diffusion coefficient become a comparatively simple matter. The coefficient may then be computed from the amount of the gas taken up during a certain period, and a representative value for the tension difference existing during this time interval.

Carbon monoxide was proposed by BOHR (1909) as being particularly suitable for determination of the diffusing capacity of the alveolar membrane. BOHR reasoned that during the initial phase of a CO uptake, there should only be an insignificant CO tension in the erythrocytes traversing the alveolar capillaries since haemoglobin has a very great affinity to CO. Consequently, in this period no significant error should be introduced by assuming the CO tension difference across the alveolar membrane to be identical with the CO tension existing in the alveolar air. Moreover, he pointed out that since CO combines with haemoglobin the "alveolar membrane" is similar in extent and nature for this gas and the respiratory gases.

BOHR's assumption of only a negligible CO tension in the erythrocytes during the initial phase of a CO uptake still lacks proof. The size of this pressure depends on the rate of reaction of CO with haemoglobin, not only on the affinity of haemoglobin for CO, which merely reflects the *ratio* between the rate constant of this and that of the inverse reaction at equilibrium.

The first extensive experimental study of the lung diffusion coefficient for CO($D_{L_{CO}}$) in human subjects was made by A. and M. KROGH (1909, 1914, 1915). Determinations were made by the latter in 22 healthy persons and in 8 patients with lung diseases. Since then $D_{L_{CO}}$ determinations seem to have received only sporadic attention, and apparently have not been explored as a tool in the study of lung disorders. It was therefore felt that such determinations deserved renewed interest.

However, since M. KROGH's technique has certain features which makes it unsuitable for determinations on patients with advanced lung diseases, it was decided to attempt a somewhat different approach.

Briefly summarized the technique of M. KROGH (1914) was the following: the subject expired maximally and then made a single deep inspiration of a measured volume of CO-containing air; immediately afterwards an expiration was performed deep enough to wash out the dead space. Then followed a pause of five to ten seconds and finally another expiration to the residual volume. At both expirations, "alveolar air" was sampled for CO analyses.

The residual volume was determined in a separate experiment. This technique has the following drawbacks:

1) Achievement and maintenance of identical carbon monoxide tension throughout the alveolar air cannot be expected following a single inspiration, and especially not in cases of lung disorders. 2) The vital capacity of many patients is too small to wash out the dead space twice, and furthermore such patients may be unable to hold their breath for a sufficiently long period. 3) The fact that the residual volume is determined in a separate experiment constitutes a potential source of error.

Consequently another procedure was chosen, using continuous rebreathing from a closed system. Thereby determinations could be made even in patients with low vital capacities, and it is felt that a more complete mixing of the alveolar air was achieved. Furthermore, C^{14} labelled carbon monoxide was used so that it became possible to determine the residual air and the carbon monoxide uptake in the same experiment. Also since in contrast to $C^{12}O$, no $C^{14}O$ tension initially exists in the blood, it was possible to use quantities of carbon monoxide so small that only a minute fraction of the total oxygen transport capacity of the blood was affected.

This experimental technique, some theoretical questions concerning the determination of $D_{L_{CO}}$, and the results obtained in healthy persons will be discussed below. Described below are also some separate experiments on rats which indicated that the rate of metabolic conversion of CO is so slow that even the repeated use of 3 to 4 μC quantities of $C^{14}O$ should involve no risk in experiments on human beings.

The results of measurements on patients with lung disorders will appear in another paper by KJERULF-JENSEN and KRUHØFFER.

Experimental Technique and Analytical Methods.

Procedure for Determination of $D_{L_{C^{14}O}}$.

Following a preliminary determination of the vital capacity, a known volume of gas mixture was introduced into a Grollman bag, generally about 1 liter less than the vital capacity. The mixture contained about 20 per cent H_2 (exact percentage known from analysis), 20 per cent O_2, the remainder being N_2. In all cases a volume of about 2 ml of carbon monoxide containing about 3.5 μC of $C^{14}O$ was introduced into the bag

together with the bulk of gases. In some cases where the effect of varying O_2 tensions on the calculated $D_{Lc^{14}O}$ was studied, other compositions of the bag air were used (O_2 percentages from 10 to 90), and the lungs were sometimes washed for a half to one minute with oxygen-rich air before the subject was connected to the bag. The upper end of the bag

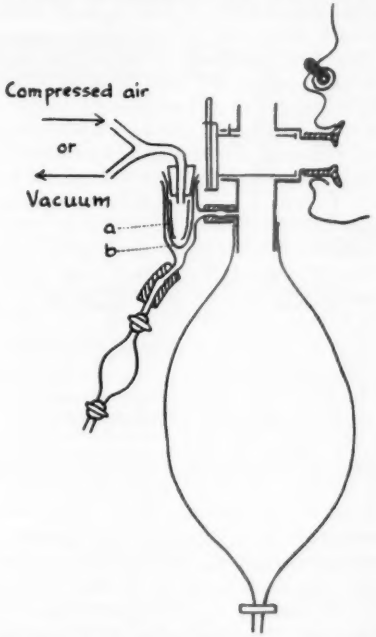


Fig. 1. *Diagram of apparatus used for breathing and sampling of gas mixtures for* $D_{Lc^{14}O}$ *determinations.*

a is a short glass tube preventing the complete collapse of the rubber cap (*b*) of the valve when vacuum is applied.

had a cock (fig. 1) which permitted connection of the subject by a mouthpiece to either the atmosphere or the bag.

Following a short period of breathing of atmospheric air the subject expired maximally; then connection was established with the bag and the subject started breathing deeply and rather fast (about 25 respirations per minute) to and from the bag, endeavouring to empty the bag completely with each inspiration.

After some 12 seconds a first sample of the air of the system was collected towards the end of an expiration, and the sampling was repeated twice, generally 20 to 23 and 30 to 35 seconds after connection to the bag was established.

The samples were drawn into pre-evacuated recipients of known volumes (ca. 20, 40, and 100 ml, respectively) through three short and narrow side-tubes on the tube connecting the bag with the cock. Sampling was effected by means of special valves, one of which is shown in fig. 1. Before sampling, the rubber cap of the valve (finger from surgical rubber glove) was kept distended by compressed air supplied

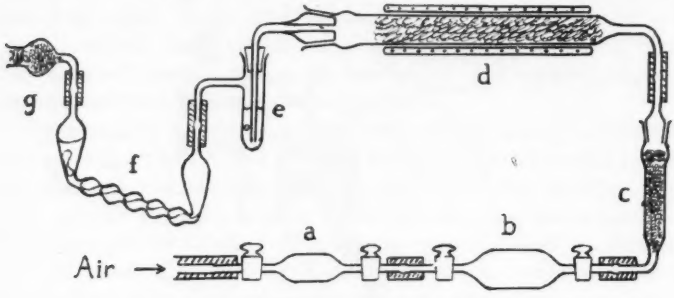


Fig. 2. *Combustion train for analysis of $C^{14}O$.*

Dry, CO_2-free air is passed through the train at constant pressure. *a*, recipient containing about 7.5 ml of inert CO. *b*, Sample recipient containing $C^{14}O$. *c*, Ascarite-filled CO_2 absorption tube. *d*, Combustion tube, 250 × 14 mm electrically heated to about 500°C and containing asbestos fiber impregnated with CuO. *e*, Tube containing 10 % $HClO_4$, for saturating the air with water vapour. *f*, Tube, containing 4 ml 2 N NaOH, for CO_2 absorption. *g*, Ascarite-filled CO_2 protection tube.

to it through one rubber tube. Sampling was made by pressing down a lever, thereby simultaneously closing this tube and releasing compression from another supplying vacuum; by the resulting collapse of the rubber cap, communication was established between the inlet and the outlet tube of the valve. The depression of the lever also lead to closure of an electric circuit which actuated a signal magnet. Thus, the time of sampling could be recorded, together with a time marking on a smoked drum.

Analytical Methods.

A sample of gas withdrawn from the bag at the end of the experiment was analysed for CO_2, O_2 and H_2 in a Haldane gas analysis apparatus.

The total amount of C^{14} radioactivity present in CO in each recipient was determined by conversion of CO to CO_2 which was subsequently precipitated as $BaCO_3$ on filter paper disks and counted with a thin end-window counter. Due to the increasing volume of the recipients, the counting rates for all disks were 10 to 25 times the background counting rate (ca. 20 counts per min.). At least 12,288 counts were registered from each disk.

The amount of $BaCO_3$ was kept close to 16 mg on all disks to increase the accuracy of the counting. To achieve this it was necessary to remove the CO_2 present in the recipient before the combustion, and to combust together with the CO in the recipient a known amount of inert CO. The combustion procedure was therefore carried out in trains of vessels like the one shown in fig. 2, which explains the details. Air (or O_2) was blown through the train with somewhat increasing speed (1 to 5 bubbles per sec.) during 1 to $1^1/_2$ hour. The CO_2 leaving the combustion tube was absorbed in 4 ml of 2 N NaOH. Two 1 ml aliquotes of this solution were used for preparation of $BaCO_3$ disks, using a filtering device similar to the one described by HENRIQUES et al. (1946) ($BaCO_3$ area 2.7 sq.cm). Self absorption corrections were applied which converted all counts to the common basis of a 16 mg plate weight. From the figures obtained, the counts/min./ml of sampled air were calculated (a_1, a_2 and a_3).

The joint accuracy of the sampling, combusting and plating procedures was tested by filling three recipients with the same $C^{14}O$ containing air, and carrying out the analytical procedure as outlined. In four tests the average values of three $BaCO_3$ platings made from the CO_2 obtained from each recipient never differed more than 3 per cent from the general average for all 3 recipients.

Preparation of $C^{14}O$.

$C^{14}O_2$ generated from $BaC^{14}O_3$ was reduced over heated Zn using a modification of BERNSTEIN and TAYLOR's (1947) procedure. The apparatus is shown in fig. 3. 25 mg $BaC^{14}O_3$ (~ 350 μC) + 1.96 g of inert $BaCO_3$ was placed in the vial *a*. The whole system was then alternately evacuated and filled with N_2 several times from *i* and finally evacuated to less than $^1/_{10}$ mm Hg, leaving the mercury in *f* just below the double oblique bore stopcock *e*. The tube *d* (150 × 20 mm) containing zinc dust-asbestos fiber pellets was heated to 400°C, by its electric heating mantle. With stopcock *b* open and *e* closed 40 per cent $HClO_4$ was dropwise added from *c* until barometric pressure had developed in *a*. After a pause of 10 minutes *e* was opened towards the left with the mercury in *g* slightly below the level in *f* and while maintaining a slight sub-barometric pressure in *f* slow dropwise addition of $HClO_4$ was continued over $^3/_4$ hour until *a* was completely filled up. Stopcock *b* was then closed and the CO generated transferred to *h* (until then filled with Hg) by means of the Hg-pump *f*—*g*. Traces of non-reacted $C^{14}O_2$ were removed from the gas by passing it several times to and from a NaOH filled absorber. The yield of 210 ml (0°, 760 mm) was about 95 per cent of the theoretical. Subsequently *h* was used for storing and dispensing $C^{14}O$. The volume between its stopcocks (2.2 ml) was evacuated and then filled with $C^{14}O$ from the main container this $C^{14}O$ then being transferred to the bag by the stream of bulk gases entering at *i* and leaving at *k*. The volume of 2.2 ml (at 760 mm and room temperature) generally employed in the experiments con-

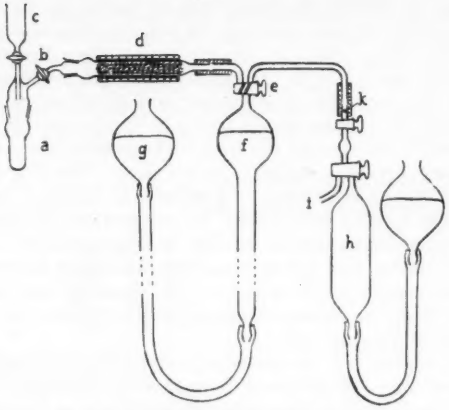


Fig. 3. *Apparatus for generation of $C^{14}O$.*
Description in text.

tained about 3.5 μC of C^{14} corresponding to about 600,000 counts/min. with the counting arrangement in use (and referred to the standard plate weight of 16 mg $BaCO_3$).

Calculation of $D_{L_{C^{14}O}}$.

The data needed for the calculation are: 1) V, the volume of dry gases introduced into the bag, given at 30° C, and barometric pressure, P_B; 2) the initial H_2 percentage (h_g) in the dry gases of this volume; 3) the H_2 percentage (h_m) of the mixed dry gases of the closed system at the end of the experiment; 4) the amount of radioactivity per ml of air a_1, a_2 and a_3 (counts/min./ml) in at least two samples of air from the closed system and 5) the times of withdrawal of these samples t_1, t_2 and t_3 (in seconds). Three assumptions, which are commented upon below, are made in the calculation:

1) At the withdrawal of the first sample and subsequently, the air in the closed system (lungs, connections, bag) is completely mixed.

2) The total gas volume of the closed system is unchanged throughout the experimental period.

3) At any time of the experiment the $C^{14}O$ tension in the erythrocytes in the lung capillaries is negligible in relation to that existing in the alveolar air.

If so the volume of $C^{14}O$ at P_B mm Hg and 30° $= {}^{30}_{P_B}D_{L_{C^{14}O}}$, taken up per second per mm of alveolar $C^{14}O$ tension will be

$$ {}^{30}_{P_B}D_{L_{C^{14}O}} = \frac{\frac{dP_{C^{14}O}}{P_B} \cdot S}{dt \cdot P_{C^{14}O}} \qquad \ldots\ldots\ldots \quad (1) $$

Here S is the volume of the closed system (water vapour saturated, P_B mm Hg, 30° C); 30° C is the average temperature of air in lungs and bag, P_B is barometric pressure in mm Hg, $P_{C^{14}O}$ is the $C^{14}O$ tension existing in the closed system at one particular time, and $dP_{C^{14}O}$ is the change occurring in dt seconds.

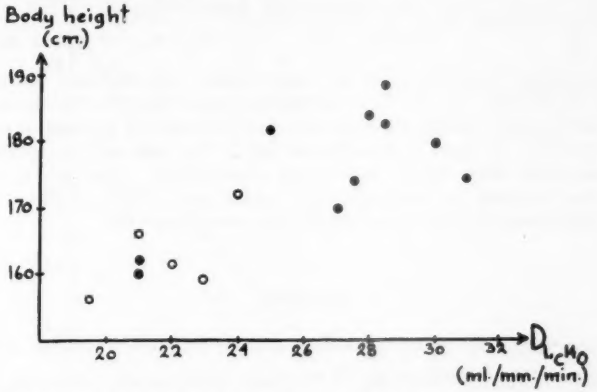


Fig. 4. *Relation between body heights and values of* $D_{L_{C^{14}O}}$ *obtained at* O_2 *percentages of about 15.*

Open circles, women; filled circles, men.

Integration between the limits, $C^{14}O$ tension P_1 at time t_1 and P_2 at time t_2 gives

$$^{30}_{P_B}D_{L_{C^{14}O}} = 2.303 \cdot \frac{S}{P_B} \cdot \frac{\log P_1 - \log P_2}{t_2 - t_1} \quad \ldots\ldots (2)$$

Substituting for P_1 and P_2 the radioactivity concentrations a_1 and a_2 (which are proportional to them) and converting to standard conditions (760 mm, 0°) gives

$$^{0}_{760}D_{L_{C^{14}O}} = 2.303 \frac{S}{760} \cdot \frac{\log a_1 - \log a_2}{t_2 - t_1} \cdot \frac{273}{273 + 30} \quad \ldots (3)$$

S in this equation is obtained from the determinations of the residual volume.

On the above assumptions (1 and 2) and assuming furthermore that the total amount of H_2 in the system remains unchanged during the experiment, the dry residual volume, R (= air in lungs and connections), at P_B mm and 30° is given by

$$V \cdot h_g + R \cdot O = (V + R)h_m \text{ or } R = V \cdot (h_g - h_m)/h_m.$$

From this the volume at P_B mm and 30° of dry gases in the closed system is $= R + V = V(1 + (h_g - h_m)/h_m)$, and assuming an average

water vapour tension of 35 mm in the closed system, S in the above equation (3) becomes

$$S = V(1 + (h_g - h_m)/h_m) \cdot P_B/(P_B - 35).$$

Thus the complete equation used for calculation of $D_{Lc^{14}O}$ was

$${}^{0}_{760}D_{Lc^{14}O} = 2.303 \frac{V(1 + (h_g + h_m)/h_m) \cdot P_B}{760(P_B - 35)} \cdot \frac{\log a_1 - \log a_2}{t_2 - t_1} \cdot \frac{273}{273 + 30} \quad (4)$$

The results given below are on a one minute basis and were obtained by multiplication by 60. The calculations were made on the basis of the first and last sample. Withdrawal of an intervening (second) sample was included to check a logarithmic fall in the radioactivity concentration with time. Those rather few experiments where a_1, a_2 and a_3, when plotted on semilogarithmic paper as function of time, did not fall reasonably well on a straight line were discarded.

Results.

Determinations of $D_{Lc^{14}O}$ were made on 15 resting healthy adult persons of ages between 24 to 60 years using an O_2 percentage of about 15 per cent in the closed system. The results and their relation to the height and sex of the subjects are shown in figure 4. A correlation with height is evident but the number of observations is too small to provide a basis for a closer formulation.

The average value (22 ml/min./mm Hg) obtained for 5 women is lower than the average (27 ml/min./mm Hg) obtained for 10 men, but it appears that this may essentially be accounted for by differences in body heights. These values agree well with those of M. KROGH (1914); namely 21 as an average for 3 women and 29 as an average for 14 men.

Repeated determinations on the same subjects were carried out in 6 cases; the results obtained indicated a surprisingly good reproducibility. Deviations above 10 per cent from the mean were not observed, even when several months elapsed between the determinations. A similar reproducibility was observed by M. KROGH.

A rather thorough study was made in two persons on the effect of different oxygen tensions in the air of the closed system on the $D_{Lc^{14}O}$ values as calculated according to formula (4) above. These two persons happened to give almost identical $D_{Lc^{14}O}$ values at similar O_2 tensions. The results are plotted in fig. 5.

It is apparent that within the range of oxygen tensions from

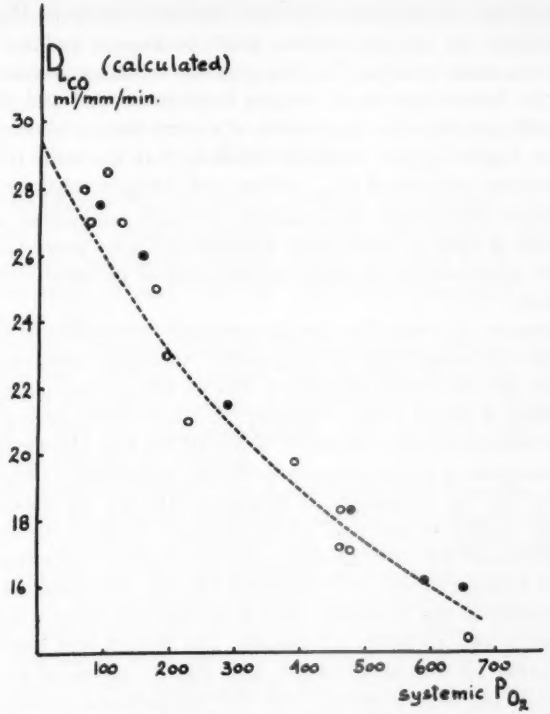


Fig. 5. *Relation for two persons between the oxygen tension in the closed system and the* $D_{Lc^{14}O}$ *values as calculated from formula (4), i. e. disregarding CO tension in the erythrocytes.*

The oxygen tension was calculated as $\frac{(B\text{-}47)\cdot(O_2^f+2)}{100}$ where O_2^f is the O_2 percentage of the dry air in the bag at the end of the experiment. The stipled line represents theoretical D_{LCO} values calculated from the equation

$$D^x_{L_{CO}} = \frac{3.6+1}{3.6+x/200}\cdot 23,$$ where x means any systemic oxygen tension.

about 100 to 650 mm, the calculated $D_{Lc^{14}O}$ values are inversely related to the oxygen tension.

At oxygen tensions below about 100 mm Hg the calculated $D_{Lc^{14}O}$ values appear to be rather unrelated to the oxygen tension, but considering the error inherent in the method a larger number of observations would obviously be needed before a conclusion to this effect can be drawn.

A practical consequence of these findings is that in $D_{L_{CO}}$ determinations the oxygen tension must be known and its effect taken into consideration. For the present, it seems preferable to make the determinations at oxygen tensions around and slightly below 100 mm, since the application of a correction on observations made at higher oxygen tensions demands that the same relationship between calculated $D_{L_{CO}}$ values and oxygen tensions apply to different individuals. This has not yet been established, and in particular it may be questioned whether the same correction factors are applicable to healthy persons and to patients with lung disorders.

Evaluation in rats of a possible metabolic conversion of CO. To evaluate the magnitude of a possible metabolic conversion of CO two rats of a total weight of 400 to 450 g were placed in a desiccator of about 5 liter capacity, in which some drying agent and a dish containing 150 ml 10 N NaOH for CO_2 absorption had been inserted. A 3-hole stopper in the lid permitted: 1) replenishment of the O_2 consumed, 2) mixing of the air by means of a mercury sealed stirrer and 3) withdrawal of small air samples into recipients for $C^{14}O$ analysis. At the start of the experiment a small amount (2 ml) of C^{14} labelled CO was introduced into the atmosphere of the desiccator and 3 air samples were withdrawn between 3 and 19 hours afterwards. The NaOH was transferred to a 2,000 ml volumetric flask and $BaCO_3$ plates of a weight around 16 mg were prepared from this solution and counted.

In two such experiments it was found that after an experimental period of 19 hours 5 and 6.5 per cent respectively of the C^{14} activity introduced as CO was recovered as CO_2. (In a control experiment, without rats, the recovery was < 0.2 per cent.) Between 3 and 19 hours a drop of similar magnitude was observed in the $C^{14}O$ content of the air; however, due to the errors involved in the $C^{14}O$ determinations, it is impossible to say whether the CO metabolized was essentially converted to CO_2. Thus it is evident that the rat (and the bacterial flora of its intestines?) is capable of slowly converting CO into CO_2.

The rate of $CO \rightarrow CO_2$ conversion observed in these experiments namely somewhat less than 0.02 cu.mm/g of body weight/hour, is more than 10 times less than the rate obtained by CLARK (1950) in similar experiments in mice. On the other hand it is considerably higher than the rather uncertain values obtained by TOBIAS et al. (1945) by means of C^{11} labelled CO in human subjects.

Considering the slowness of $CO \rightarrow CO_2$ conversion indicated by the above experiments it was felt that even the repeated use of 3 to 4 μC quantities of $C^{14}O$ would be far below tolerance dosage.

Discussion.

In the calculation of the $D_{Lc^{14}o}$ from the experimental data obtained by the present technique certain assumptions had to be made. In evaluating the *sources of error* involved in the method, attention must be paid to the fact that these assumptions are not completely valid.

Obviously, complete mixing of the air in the closed system at any time has not been attained. The problem of mixing has two aspects: 1) incomplete mixing of the alveolar air as a whole with the air in the bag and connections, and 2) incomplete mixing within the alveolar air volume.

With regard to question one it is evident that since the uptake of CO takes place from the alveolar air, its CO concentration will always be somewhat lower than that of the bag and the connections. Consequently CO will be taken up somewhat slower than if mixing with the air in the bag had always been complete. However, by making a calculation from a set of simplifying assumptions which reasonably well portrays a typical experiment it can be shown that the error introduced by this type of incomplete mixing is rather small. The following assumptions were made: the size of the true $D_{Lco} = 0.45$ ml/mm/sec.; the volume of the closed system = 5 liters; the in- and expiration are of the "square" type, each of a duration of 1.5 sec. and of a magnitude of 2 liters; the amount of air left in bag + connections + dead space at the end of each inspiration is 0.5 liter; occurrence of complete mixing within the alveolar air and within the remainder of the air in the closed system. A calculation based on these assumptions shows that the measured D_{Lco} would come out some 5 per cent lower than if complete mixing had occurred in the closed system as a whole.

A quantitative evaluation of the error introduced from incomplete mixing within the alveolar air is not possible. However, it is felt that the continuous deep and rather fast breathing employed in the present procedure should be rather effective in levelling off concentration differences in various parts of the alveolar air.

The assumption of a constant systemic volume throughout the experimental period is not completely valid either, since the amount of O_2 lost from it exceeds the amount of CO_2 added. In particular this is so towards the end of the experiment when the CO_2 tension in the system approaches the tension in the mixed venous blood. Consequently, there is continuous fall in the systemic volume during the experimental period. This introduces errors in two ways: by making the calculated volume of the closed system (S) too small (h_m is estimated too high), and by reducing the drop in radioactivity concentration (a_1—a_2). Since under resting conditions the volume of O_2 taken up in the experimental period ($t_2 - t_1$) does not exceed the volume of CO_2 simultaneously given off to the system by more than 100 ml, it can be estimated that the error introduced in the $D_{L_{CO}}$ determination by this factor hardly exceeds 5 per cent.

The validity of the assumption of an essentially negligible $C^{14}O$ tension in the erythrocytes of the alveolar capillaries may also be questioned. The only reasonable explanation of the fall in the *calculated* $D_{L_{C^{14}O}}$ values with increasing systemic O_2 tension (cf. fig. 5), is that with increasing systemic O_2 tensions the rate of reaction of CO with the haemoglobin is slowed down as a result of which an increasing CO tension develops in the erythrocytes. The same effect of increasing oxygen tensions has been observed in prolonged CO-uptake experiments by FORBES, SARGENT and ROUGHTON (1945). The decrease in the rate of reaction of CO with haemoglobin at increasing O_2 tensions has been thoroughly studied by ROUGHTON (1934, 1945) in *in vitro* experiments on sheep and human blood. It was found that over its first half the reaction $CO + HbO_2 \rightarrow HbCO + O_2$ could be described by the equation $d[HbCO]/dt = m' \cdot \frac{[CO][HbO_2]}{[O_2]}$. In other words, in the range of high O_2 tensions the rate of reaction of CO with haemoglobin should be inversely proportional to the O_2 tension to which the haemoglobin is exposed.

Thus it seems beyond doubt that an appreciable CO tension has existed in the erythrocytes at high oxygen tensions. The question remains whether it is permissible to assume a negligible CO back tension at low oxygen tensions, or stated otherwise, whether the $D_{L_{CO}}$ values observed at such conditions are significantly lower than the true values, *i. e.* those values which would have been

obtained if a CO tension of zero had existed in the erythrocytes during the uptake. Measurements of the $D_{L_{CO}}$ values at different levels of oxygen tension together with knowledge of the ratio of the reaction rates of CO with haemoglobin at these levels should allow a calculation of the true $D_{L_{CO}}$ and thus be capable of answering this question.

Let $D^t_{L\text{-}CO}$ designate this "true" $D_{L\text{-}CO}$, which is the rate (ml/min.) at which CO would have diffused from the alveolar air to its site of reaction with haemoglobin in the erythrocytes if one mm of CO tension difference had existed between these places.

Let further C_a be the rate (ml/min.) at which CO would have formed HbCO in the erythrocytes traversing the alveolar capillaries had the reacting haemoglobin been directly exposed to one mm of CO tension and to a certain oxygen tension indicated by subscript a. Also, let C_b be correspondingly defined but at a different oxygen tension (indicated by subscript b).

Finally, let $D^a_{L\text{-}CO}$ and $D^b_{L\text{-}CO}$ designate calculated values of $D_{L_{CO}}$ (ml/min./mm) obtained from experiments at the (erythrocyte) levels of oxygen tension a and b.

Since both the C's and the D_L's are comparable to electric conductances and their reciprocals to resistances:

$$\frac{1}{D^b_{L\text{-}CO}} = \frac{1}{D^t_{L\text{-}CO}} + \frac{1}{C_b} \text{ and } \frac{1}{D^a_{L\text{-}CO}} = \frac{1}{D^t_{L\text{-}CO}} + \frac{1}{C_a}.$$

$$\text{If we define } \frac{C_b}{D^t_{L\text{-}CO}} = n \text{ and } \frac{C_b}{C_a} = m \text{ we get}$$

$$\frac{1}{D^b_{L\text{-}CO}} = \frac{1}{D^t_{L\text{-}CO}} + \frac{1}{n \cdot D^t_{L\text{-}CO}} \text{ and } \frac{1}{D^a_{L\text{-}CO}} = \frac{1}{D^t_{L\text{-}CO}} + \frac{1}{n \cdot D^t_{L\text{-}CO}/m}$$

$$\text{or } D^b_{L\text{-}CO} = \frac{n}{n+1} D^t_{L\text{-}CO} \text{ (1) and } D^a_{L\text{-}CO} = \frac{n}{n+m} D^t_{L\text{-}CO} \text{ (2), from which}$$

$$\frac{D^a_{L\text{-}CO}}{D^b_{L\text{-}CO}} = \frac{n+1}{n+m} \text{ (3).}$$

With $D^a_{L\text{-}CO}$ and $D^b_{L\text{-}CO}$ experimentally determined and knowledge of the size of m at the corresponding oxygen tension levels, n can be calculated from (3) and subsequently $D^t_{L\text{-}CO}$ from (1) or (2).

Now according to ROUGHTON's findings, cited above, if a and b

are chosen sufficiently high, m, the ratio C_b/C_a should be equal to the reverse ratio of the existing oxygen tensions: *i. e.* $= \frac{a}{b}$. If from fig. 5 we take $D^a_{L_{CO}} = 16$ at a about 600 mm, and $D^b_{L_{CO}} = 23$ at b about 200 mm we get $m = 3$ and thus $n = 3.6$ and $D^t_{L_{CO}} = 29.4$. This figure for the true $D_{L_{CO}}$ is some 7 per cent larger than the $D_{L_{CO}}$ values obtained at such low systemic oxygen tensions (< 100 mm), which would permit considerable amounts of reduced haemoglobin to exist in the erythrocytes of the alveolar capillaries. Thus these calculations indicate that under such conditions the CO tension in the erythrocytes should be of almost negligible magnitude. However, it must be admitted that certain objections may rightly be raised against the above calculations. Thus it may be objected that the sites of reaction of CO with haemoglobin may not be identical at different levels of oxygen tension, making it incorrect to ascribe a fixed value to $D^t_{L_{CO}}$.

Further, it is true that the two (P_{O_2}, $D_{L_{CO}}$) co-ordinate pairs, chosen as a basis for the calculations are not sufficiently well-defined to permit a very accurate calculation of the $D^t_{L_{CO}}$. A more accurate value for the latter could undoubtedly have been obtained if instead of the co-ordinate pair (200 mm, 23 ml/min./mm) a pair representing an oxygen tension below 100 mm Hg could have been used. This however was not possible, since no data are available on the ratio between the reaction rate of CO with haemoglobin at oxygen tensions well below 100 mm Hg and that at a high oxygen tension.

In fig. 5 a stipled line is drawn through values calculated from the equation $D^x_{L_{CO}} = \frac{3.6 + 1}{3.6 + x/200} \cdot 23$ derived from the above pair of values; x here means any oxygen tension and $D^x_{L_{CO}}$ the $D_{L_{CO}}$ value which one should obtain at this tension if (as is not to be expected) ROUGHTON's finding of a reverse relationship between the reaction rate of CO with haemoglobin and the oxygen tension held for all oxygen tensions. At oxygen tensions above 200 mm the agreement with the experimentally determined values is excellent. At values below about 200 mm the experimentally determined $D_{L_{CO}}$ values tend to be somewhat higher than those read from the curve at corresponding oxygen tensions. This in fact is to be expected since in this range reduced haemoglobin,

with which CO reacts much faster than with HbO_2, will occur in the alveolar capillaries in perceptible concentrations.

It can easily be shown by calculation that the $C^{14}O$ tension developed in the alveolar capillaries from *recirculation* of $C^{14}O$ is of no significance, for the experimental periods used in these studies.

From the above discussion it may be concluded that several factors tend to make the $D_{L_{CO}}$ values determined by the present procedure app. 10 to 15 per cent lower than the true values.

ROUGHTON (1945) has made a calculation of the average time spent by the blood in the alveolar capillaries on the basis of experimental observations of the decrease in *rate of CO uptake* in the blood with increasing alveolar oxygen tension in persons inhaling CO containing gas mixtures. However, at least in experiments during rest with a low alveolar ventilation such rates of CO uptake are not directly comparable for this purpose, since they will have occurred at different alveolar CO tensions even when the same CO percentage was present in the inspired air. More correct figures should be computable from the above mentioned calculated $D_{L_{CO}}$ values obtained at different oxygen tensions. Such calculations[1] lead to values for the average time (during rest) which are some 100 per cent larger than those obtained by ROUGHTON.

Ever since determinations of the lung diffusion coefficient were introduced, the problem has been discussed whether the values obtained were sufficiently high to account by simple diffusion for the rates of oxygen uptakes observed, especially during exercise and at reduced oxygen tensions. It was therefore originally intended to make $D_{L_{CO}}$ determinations during heavy exercise. It was soon realized, however, that the present technique, as well as

[1] According to the present data an alveolar CO tension of 1 mm would lead to an uptake of 29.4 ml of CO per minute if no CO tension existed in the erythrocytes. At an O_2 tension of 600 mm only an uptake of 16 ml per minute is observed; thus under these conditions a CO tension of $(29.4 - 16)/29.4 = 0.45$ mm should have existed in the erythrocytes. Assuming the individuals to have a total of 800 g of haemoglobin, exposition of all of it to a P_{CO} of 0.45 mm and a P_{O_2} of 600 mm would according to ROUGHTON'S formula (given above) lead to the formation of $21 \cdot \frac{0.45}{600} \cdot 0.77 \cdot 800 \cdot 60 = 585$ g of HbCO per minute. (0.77 is the ratio between the solubilities of CO and O_2). Actually only $16/1.34 = 11.9$ g would be formed. Consequently, one obtains a ratio of $11.9/585 = 0.020$ between the average time spent by erythrocytes in the alveolar capillaries and the average circulation time for erythrocytes (t_L/t_B in ROUGHTON'S terminology). This is some 100 per cent higher than the value obtained by ROUGHTON and this also applies to the calculated average time spent by the blood in the alveolar capillaries during rest.

that of M. KROGH, were useless for such determinations since much too low figures would be obtained due to the fact that during the experimental period a marked reduction would occur in the air volume from which CO is removed (*v.s.*). In one experiment it was attempted to maintain a constant systemic volume by absorbing the CO_2 and replenishing the O_2 used by continuous inflow in the bag. At a working rate of 1,080 kgm/min. a $D_{Lc^{14}o}$ of 45 to 50 was obtained (resting control 28).

However, these studies were not continued, partly because it was felt that the procedure was technically unsatisfactory for this purpose, and partly because it seems doubtful whether even correct $D_{L_{co}}$ determinations could solve the question whether oxygen uptake is entirely due to diffusion. To answer this question it is necessary to have not only a correct determination of the lung diffusion coefficient for oxygen (computable from the $D_{L_{co}}$) but also to be able to determine correctly the *weighted* mean oxygen tension difference between "alveolar air" and the erythrocytes. However, the only method available for this purpose, the graphical integration method of CHR. BOHR (1909), depends on the assumptions that all alveolar capillaries are equi-dimensional and equally perfused, conditions which are almost certainly not realized in the lungs.

The occurrence of a considerable rise in the $D_{L_{co}}$ values of healthy subjects during exercise indicates a marked expansibility of the alveolar capillary bed. It is likely that this functional reserve is much smaller in patients with advanced pulmonary disorders. $D_{L_{co}}$ determinations performed during such work which causes maximal mobilization of the reserve would therefore be of considerable clinical interest, and the development of simple, accurate procedures for this purpose should be welcomed.

Summary.

A technique for determinations of the lung diffusion coefficient for carbon monoxide ($D_{L_{co}}$) in resting subjects has been developed. Following deep expiration, the subject breathes continuously to and from a Grollmann bag initially filled with a known volume of a gas mixture containing a known percentage of H_2 and some C^{14} labelled CO. Within about 35 seconds 3 samples of the air in the closed system are withdrawn for determination of the $C^{14}O$ concentration.

On the basis of the final H_2 percentage, the volume of the closed system is obtained and from this and the rate of decrease in radioactivity concentration, the $D_{Lc^{14}o}$ is calculated.

The procedures for preparation and analysis of $C^{14}O$ are described. The results of $D_{Lc^{14}o}$ determinations in 15 healthy adult subjects at an O_2 percentage of about 15 in the closed system are given. A correlation with body height was observed and in the main appears responsible for the higher average (27 ml/min./mm) observed in men than in women (22 ml/min./mm).

The decrease in the calculated $D_{Lc^{14}o}$ caused by increasing the systemic oxygen tension was studied in two subjects, and was interpreted on the basis of ROUGHTON's finding that the rate of reaction of CO with haemoglobin was inversely proportional to the oxygen tension at high levels of the latter. Also, on this basis, a calculation of the "true" D_{Lco} was attempted; the figure obtained differed little from those obtained experimentally at oxygen tensions below 100 mm, indicating an almost negligible CO tension in the erythrocytes traversing the alveolar capillaries at such oxygen tensions.

The errors of the present procedure are discussed, and the conclusion drawn that together they will make the observed D_{Lco} values roughly 10 to 15 per cent too low.

The conversion of $C^{14}O$ to $C^{14}O_2$ was studied in rats and found to be slow (< 0.02 cu.mm/g body weight/hour when 2 rats were enclosed into a space of 5 liters initially containing 2 ml of $C^{14}O$).

References.

BERNSTEIN, R. B. and T. I. TAYLOR, Science 1947. *106*. 498.

BOHR, C., Skand. Arch. Physiol. 1909. *22*. 221.

CLARK, R. T., Amer. J. Physiol. 1950. *162*. 560.

FORBES, W. H., F. SARGENT and F. J. W. ROUGHTON, Ibidem 1945. *143*. 594.

HENRIQUES, F. C., Jr., G. B. KISTIAKOWSKY, C. MARGNETTI and W. C. SCHNEIDER, Ind. and Eng. Chem., Anal. Ed. 1946. *18*. 349.

KJERULF-JENSEN, K. and P. KRUHØFFER. Acta med. scand. (In press.)

KROGH, A. and M. KROGH, Skand. Arch. Physiol. 1910. *23*. 263.

KROGH, M., J. Physiol. 1915. *49*. 271.

— Luftdiffusionen gennem Menneskets Lunger. Thesis. Copenhagen 1914.

ROUGHTON, F. J. W., Proc. Roy. Soc. B. 1934. *115*. 473.

— Amer. J. Physiol. 1945. *143*. 609 and 621.

TOBIAS, C. A., J. H. LAWRENCE, F. J. W. ROUGHTON, W. S. ROOT and M. I. GREGERSEN, Ibidem. 1945. *145*. 253.

From the Research Laboratory, Department of Gynaecology, Sabbatsberg Hospital and Chemistry Department II, Karolinska Institutet, Stockholm, Sweden.

An Autoradiographic Study on the Occurrence of Injected Radiosulphate in the Intestine.

By

HARRY BOSTRÖM and ERIK ODEBLAD.

Received 10 April 1954.

It was pointed out several years ago (ODEBLAD and BOSTRÖM 1952 a, b) that radioactive sulphate has a great affinity to the epithelial lining of the intestinal mucosa and that it is taken up to a considerably less extent in the other layers of the intestine. These observations have recently been confirmed by two groups of works (BELANGER 1953, DAVIES and YOUNG 1954). The present investigation is a time-relation study of the S^{35} fixation in the intestine.

Experimental

The main experimental series consisted of 27 rabbits; they were given 0.65 μC of S^{35}-labelled sodium sulphate per g of body weight as an intravenous injection. The animals were killed by air embolism in groups of three, 2, 4, 8 and 16 hours, 1, 2, 5, 10 and 16 days respectively after the injection. Pieces of the intestine were fixed in pure methanol and embedded in paraffin; they were then cut into sections 10 μ thick. The sections were mounted on methacrylate slides and exposed on Gevaert Dentus Rapid film (X-ray film) for periods ranging from 3 to 10 weeks. The films were developed in Kodak DK 20 with KBr added to 0.2 per cent for 20 minutes at 18.3° C. After fixing, washing and drying and removal of the silver on the reverse side with Farmer's solution, the films were examined and compared with the histological sections, stained with haemalum-eosin.

Additional observations were made on mice. They were given subcutaneous injections of 60 μC of S^{35}-labelled sulphate per g of body weight. Five mice were killed (with ether) after 2 days and five after 21 days.

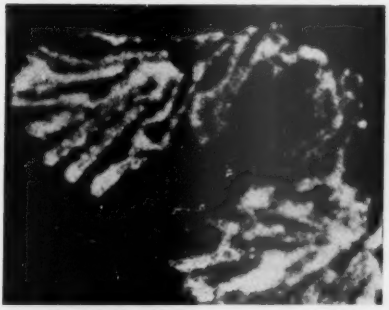


Fig. 1. Enlargement of a part of an autoradiograph of the intestine of a rabbit given radioactive sulphate 2 hours before death. A very high, more or less even accumulation of S^{35} is visible in the epithelial lining. In all the figures, the radioactivity is indicated by white areas. The magnification is 40 × throughout.

After fixing in methanol and embedding in paraffin, the specimens were sliced into 4 μ thick sections, which were mounted on glass slides, covered with Kodak autoradiographic plate stripping film and exposed for 24 or 40 days. After development in Kodak D-19-b for 20 minutes at 18.3° C, fixing, washing and drying, the sections were stained through the film with acid polychrome methylene blue.

Results.

On examination of the X-ray film autoradiographs it was found that during the first 2—4 hours after injection, there was a very high incorporation of radioactive sulphate with a more or less even distribution (within the resolution of the technique) in the epithelial lining of the intestinal mucosa (fig. 1). Eight and sixteen hours after the injection, the radioactivity appeared more concentrated in spots, which corresponded to droplets of mucus in the intestinal epithelium (fig. 2). After still longer intervals (24 hours and 2 days after the injection) the spots of high activity showed a marked tendency to be less common, and 5 days after injection practically none were visible. This gradual disappearance of the radioactive droplets of mucus is illustrated in figs. 3 and 4. In this connexion it may be mentioned that the intestinal contents, when visible, often contained large amounts of S^{35}, especially 1 and 2 days after the injection.

Five days and later the intestinal epithelium presented a different picture. The spots of high activity corresponding to the

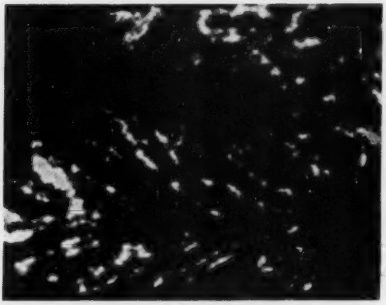


Fig. 2. Picture 16 hours after injection. Typical spotty incorporation of radioactive sulphate. The spots correspond to droplets of mucus in the epithelial cells.

droplets of mucus were replaced by a weak accumulation of radiosulphate, apparently diffuse over the whole or the inner parts of the epithelial lining. The amount of radioactivity declined from 5 to 10 days after the injection and was still lower 16 days after it. This type of accumulation is shown in fig. 4.

The propria and the submucosa contained small amounts of radiosulphate at all the intervals studied. The uptake of the muscular layers was greater than that of the submucosa, but usually considerably less than that of the epithelium (figs. 3 and 4). The serosa sometimes contained more radioisotope than the muscular layers, especially 4—16 hours after the injection.

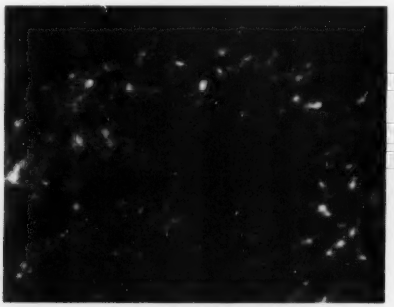


Fig. 3. Picture 2 days after injection. The number of spots is considerably reduced. The weak activity in the epithelium is visible in addition to the spots.

Fig. 4. Picture 5 days after the injection. The spotty distribution of radioactive sulphate is almost entirely lacking and only the weak diffuse accumulation remains in the epithelium. The submucosa is also seen, almost completely devoid of S^{35}. The muscularis contains more S^{35} than the submucosa but less than the epithelium.

Examination of the stripping film autoradiographs from mice 2 days after injection clearly showed that the spots of high radioactivity corresponded exactly to the mucus droplets, which stained metachromatically. When the autoradiographs were studied 21 days after injection, no such activity over the droplets could be detected. Comparatively weak activity was observed in the cell nuclei, as described earlier (ODEBLAD and BOSTRÖM 1953). The activity was weak and diffuse in the epithelial cells, connective tissue and muscular tissue.

Discussion.

The present investigation has shown the existence of at least two types of incorporation of radioactive sulphate in the intestinal mucosa. There was an intense accumulation in the droplets of mucus in epithelial cells, as well as a weak accumulation more diffusely distributed over the epithelial lining. The biological half-lives appear to differ in the two types of incorporation. The spotty accumulation seemed to be associated with a half-life of a few days only since it had almost completely disappeared after 5 days. The weak, diffuse accumulation, on the other hand, apparently had a biological half-life amounting to about one week or so.

The occurrence of S^{35} in the intestinal lumen suggests that the radioactive droplets of mucus are secreted in the contents of the

gut. If this is true, the value of the biological half-life of the accumulation in the droplets may provide some information regarding the rate of secretion of mucus from the epithelial lining.

Many investigations on the sulphate exchange in various kinds of soft tissue have been made by different groups of workers (for recent reviews, see BOSTRÖM 1953, BOSTRÖM and JORPES 1954). They afford strong support to the view that radioactive sulphate is bound to a great extent to esterified muco-polysaccharides. It does not seem unwarranted to conclude that a similar mechanism is operative here, since an exact correlation between radioactivity and metachromatically staining mucus was found in mice at an interval after the injection (2 days) when inorganic radiosulphate was almost entirely eliminated from the tissue.

Summary.

Autoradiographic observations are made on the incorporation of S^{35}-labelled sulphate in the intestine of the rabbit. They show the presence of at least two types of accumulation, *i. e.*, an intense spotty uptake in droplets of mucus in the epithelial cells and a more diffuse, weak accumulation in the epithelial lining. The radioactive droplets seem to be rapidly (within a few days) secreted into the intestinal contents. A smaller accumulation of S^{35} is found in the submucosa, muscularis and serosa.

References.

BELANGER, L. F., Nature 1953. *172*. 1150.
BOSTRÖM, H., Arkiv f. Kemi 1953. *6*. 43.
BOSTRÖM, H. and E. JORPES, Experientia 1954 (in print).
DAVIES, D. V. and L. YOUNG, Nature 1954. *173*. 449.
ODEBLAD, E. and H. BOSTRÖM, Acta Path. Microbiol. Scand. 1952 a. *31*. 339.
ODEBLAD, E. and H. BOSTRÖM, Nord. Med. 1952 b. *48*. 962.
ODEBLAD, E. and H. BOSTRÖM, Exp. Cell Res. 1953. *4*. 482.

From the Institute of Medical Physiology, University of Copenhagen, Denmark.

The Renal Mechanism of "Dilution Diuresis" and Salt Excretion in Dogs.

By

EIGIL BOJESEN.

Received 15 April 1954.

Introduction.

It is an old observation, well established from experiments on dogs (MAGNUS 1900 and 1901) and rabbits (KNOWLTON 1911), that intravenous injection of isotonic saline or Ringer's solution leads to an immediate increase in the rate of urine flow. These early investigations were inspired by BOWMAN and LUDWIG's theory on ultrafiltrate formation in the glomeruli and by Starling's demonstration of the rôle played by the colloid osmotic pressure (C. O. P.) of the plasma proteins in maintenance of fluid balance across the capillary walls of the body (STARLING 1895) and his suggestion of its possible importance as a factor determining the size of the glomerular filtration (STARLING 1909). The increase in urine flow was attributed to a reduction in the C. O. P. of plasma and so in this period it was called "dilution diuresis". The assumption of a causal relationship between the lowering in C. O. P. and the dilution diuresis was based upon the lack of similar diuretic response to intravenous injection of blood and plasma as well as gum accacia containing saline solution with a colloid osmotic pressure of the same magnitude as plasma (KNOWLTON 1911). In agreement with the ultrafiltration theory it was also assumed in this early period that the so called "pressure diuresis" (*i. e.* the diuresis produced in isolated kidneys by increasing the perfusion

pressure) was referable to the same immediate cause as the dilution diuresis, viz. to an increase in the filtration pressure in the glomerular capillaries.[1] Evidence for this view was furnished by STARLING and VERNEY (1925) in experiments on isolated kidneys. They showed that the smaller the C. O. P. of plasma the smaller was the perfusion pressure required to obtain a certain rate of urine flow. WINTON (1937) and EGGLETON et al. (1940) have found it difficult to accept increased effective filtration pressure as the only explanation for the dilution diuresis phenomenon, because the diuretic effect of an increase in perfusion pressure is much smaller than the effect of a similar decrease in C. O. P. This however can not be accepted as a serious criticism because the vascular resistance in the kidney is by no means independent of the renal blood flow. In fact, the results from studies on these two "mechanical" diuresis constituted some of the most weighty evidence upon which CUSHNY (1917) based his filtration-reabsorption theory. Conversely, the demonstration by direct analysis of glomerular fluid that an ultrafiltrate of plasma is actually formed in the glomeruli (WEARN and RICHARD 1924, WALKER et al. 1941) gave strong support to the above mentioned explanation of the mechanical diuresis.

With the introduction of methods for determination of the filtration rate (REHBERG 1926) it became established that as much as 99 per cent of the filtered water is normally reabsorbed in the tubuli. Thus there seemed to be no reason to expect that the filtration pressure in the glomerular capillaries should be the only important factor determining the rate of formation of glomerular ultrafiltrate. In fact it might be expected that the rate of tubular reabsorption should greatly influence this rate and this view was clearly expressed by REHBERG in 1929 when he wrote: "jede Schädigung der Tubuli die eine Einschränkung in der Resorptionsfähigkeit dieser Gebilde bewirkt, auch eine sofortige Herabsetzung der Glomerulifunktion mit sich bringt". This point of view, however has generally been forgotten, as might be seen in the recent monograph of SMITH 1951 in which the glomerular filtration rate is treated as exclusively determined by the filtration pressure in the glomerular capillaries. According to this view the approximate proportionality which is always observed at low excretion percentages for water between the glomerular filtration rate and the rate of re-

[1] The average hydrostatic pressure minus the average colloid osmotic pressure of the plasma proteins in the capillaries.

absorption of water is considered the result of an automatical adjustment of the tubular reabsorption to the filtered load. However, in accordance with Rehberg's suggestion such proportionality might obviously just as well be explained by assuming that under such conditions it is the water-reabsorbing activity of the tubules which determines the glomerular filtration rate.

Studies on the glomerular filtration rate during mechanically evoked diureses would seem to offer ideal opportunities for distinguishing between these two possibilities since they enable one to evoke a large and sudden change in the forces driving the glomerular filtration. Thus if the glomerular filtration was an independent variable, large changes in the filtration rate should occur. If however, the filtration rate is determined by the rate at which water is reabsorbed in the tubules, one would expect no measurable changes to occur in the filtration rate, provided the tubular rate of water reabsorption remained uninfluenced by other factors during the experimental period. Measurements of the filtration rate during mechanical diureses have been carried out by several investigators especially in recent years. While leaving the more detailed consideration of these studies to the discussion below it may summarily be stated here that since most of the studies were undertaken with other problems in mind precautions were not always taken to avoid the influence of secondary factors (changes in renal blood flow, the blood volume and extracellular volume) on the kidneys. If such secondary factors only come into action after a certain latency it should be possible to determine the effect of changes in C. O. P. of plasma on the filtration rate by producing a sudden and large change in C. O. P. and by measuring the immediate effect on the filtration rate. However, during this period the rate of urine flow is rapidly changing, making it impossible to carry out correct clearance determinations by the classical procedure. Therefore, the previously published method (BOJESEN 1954) has been applied to a closer study of the dilution diuresis phenomenon, since it enables one to make reliable clearance determinations under such conditions, by correction for "dead space errors". Beside the inulin clearance and the rate of urine flow also the diodrast clearance (as a measure for the renal plasma flow) and the rates of excretion of sodium, potassium and chloride were followed.

From the experimental results it appears that such changes in C. O. P. of plasma which cause marked changes in the rates of

excretion of water and salt do not bring about significant changes in the filtration rate *i. e.* such which exceeds the accuracy of the method. These and other results of the present study will be discussed in relation to previous studies on the mechanical types of diuresis. It will be shown that these data in fact prove that the rate of tubular reabsorption of "salt" (sodium + anions) and water is independent of the filtered load presented to the tubules i. e. the rate at which salt is filtered in the glomeruli and the pressure at which water is presented to the tubular cells from the lumina. In other words "salt" and water are conceived as tubular Tm-reabsorption substances when such are defined as substances which are reabsorbed at a rate independent of the filtered load, provided they are subject to excretion in all nephrons. As a further consequence of this view the rate of tubular reabsorption of "salt" plus water is considered the principal factor determining the filtration rate.

Methods.

General procedure: All the experiments in this paper were performed on two female dogs, of the same size (body weight around 20 kg) and with the same kidney weight (about 100 g). They were fed on milk, bread and 100 g meat per day during the whole period of investigation ($1^1/_2$ years). Following this period they were used in some other experiments (BOJESEN 1954 table 3) and then killed. Their kidneys and urinary tracts showed neither macroscopical nor microscopical abnormalities. Usually not more than two experiments were performed per month on each of the two dogs used. The experiments proceeded as follows. After 2 or 3 control periods the dogs were given an intravenous injection of 200—300 ml of a salt solution, the crystalloid composition of which was similar to the dogs' plasma ultrafiltrate (6.7 g NaCl, 2.5 g $NaHCO_3$, 0.25 g K_2HPO_4, 0.07 g KH_2PO_4, 0.09 g K_2SO_4, 0.20 g CaC_2l, 1.2 g glucose, 0.2 g urea, 300 mg inulin and 35 mg diodrastiodine dissolved in 1 liter distilled water and saturated with CO_2). Two thirds of the solution were injected in 1 to 2 min. and the remainder was injected during the next 2 min. In a group of experiments plasma proteins from the same dog was added to this salt solution yielding a C. O. P. of 250—1,000 mm water. This plasma protein was prepared from plasma some weeks before. The plasma was dialyzed, lyophilized and stored in an ice box. In a few experiments dextran was used as the colloid as a 6 per cent solution of "Macrodex" (Pharmacia). The solutions were sterilized by filtration immediately before use. The inulin clearance (or when dextran was used as the colloid, the creatinine clearance) and the diodrast clearance were determined. The plasma concentrations of protein, Cl^-, Na^+ and of K^+ as well as the C. O. P. were measured. Furthermore, the rates of excre-

tion of the ions and of water were measured. The animals were not loaded at all with water or saline before the control periods except for the 10 ml isotonic saline in which the inulin and diodrast were dissolved for intraperitoneal injection. This injection was made about one hour before the control periods. The amount of diodrast given was 6 g, sufficient to ensure a concentration of 2 to 6 mg % diodrast iodine in the plasma in the first $1^1/_2$ hours of the experiment. This concentration is below the self depression limit for diodrast in dogs (MOUSTGAARD 1948) and consequently the clearance of diodrast is a measure of the effective renal plasma flow.

The blood samples were drawn from a hypodermic needle permanently inserted in the femoral artery during the whole experimental period and were collected under liquid paraffin in heparinized centrifuge tubes.

In 9 experiments the solution injected contained no colloid, while in 5 experiments it contained plasma proteins from the same dog, and in 2 experiments dextran. In a single experiment stored and filtered plasma was administered. In two additional experiments the procedure differed in so far as the injection of the solution (colloid free) was continued for 7 and 15 minutes in order to maintain the dilution of the plasma proteins a little longer.

The clearance technique: The technique for determination of clearances under conditions of changing diuresis and fairly constant plasma concentration of the clearance substances was described in detail in a previous paper (BOJESEN 1954). In the present work urine was collected over periods of not less than 7 minutes and the collections were timed with an accuracy of $^1/_4$ min. The expected accuracy of this procedure will be discussed below (page 138).

Chemical analysis: Inulin and creatinine were determined as described previously (BOJESEN 1954). Diodrast according to BOJESEN (1948). Chloride was titrated electrometrically according to the method of LEHMANN (1939), slightly modified by using a platinum electrode as the reference electrode. The redoxpotential of the fluid was stabilized by means of permanganate. The standard deviation of this method was 1 %. Na^+ and K^+ were determined by a flame photometer with an internal lithium standard (BERRY et al. 1946) slightly modified in this laboratory by Dr. P. KRUHØFFER. In double determinations on plasma the differences between the two determinations very seldom exceeded 1 mEq/l for Na^+ and 0.5 mEq/l for potassium. Hb % and the concentration of plasma proteins were determined by the methods of PHILIP et al. (1950). From the decrease in Hb % the percentage increase in plasma volume was calculated. The colloid osmotic pressure of plasma was determined with the method of HOLM-JENSEN (1949). The accuracy of the C. O. P. determinations was about $\pm$ 2.5 per cent. When the C. O. P. was not directly determined it was calculated from the protein concentrations by ADAIR's formula (1940). By means of determinations of C. O. P. and protein concentration on the same plasma samples it was found that the transformation constants in Adair's formula originally established for human plasma were also valid for dog plasma.

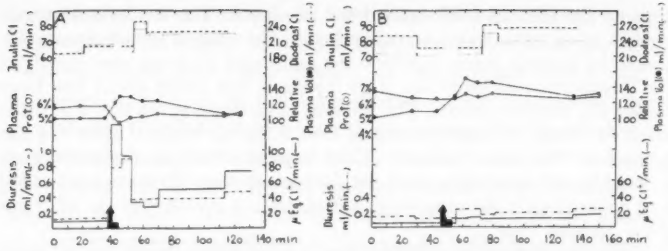


Fig. 1. Sequence of events following injections of salt solution (A) and the same solution made isotonic by means of serum proteins from the same dog (B). The effects on plasma volume, filtration rate and effective renal plasma flow are similar in the two experiments. The effect on the excretion of salt and water in the periods following the injection, however, differs strikingly in the two experiments due to the different effects on the plasma protein concentration.

The arterial blood pressure was followed throughout the experiment by direct measurements.

The latency of the dilution diuresis: A few experiments showed that after a single injection of the fluid without colloid the diuresis started 1 to $1^1/_2$ minutes after the injection and reached its maximum $^1/_4$ to $^1/_2$ min. later. Therefore in most cases, urine collection for the first post-injection period was started after the injection, the urine formed from the time of injection to this time being discarded.

In some experiments, technical difficulties made it impossible to begin the post-injection collection of urine before some 5 to 7 minutes later. In these cases the urine formed in the intervening period was collected and the changes in rate of excretion of water and "salt" arising from the changes in C. O. P. was calculated on the assumption that no changes at all occurred in the first 1 to $1^1/_2$ minutes following the injections. Clearance determinations were never attempted for these periods since corrections for dead space errors could not be applied within a reasonable degree of accuracy in these cases.

Results.

In fig. 1 A is shown the sequence of events in a typical experiment when colloid-free solution was injected.

It is seen that the lowest protein concentration was obtained in the first blood sample following the injection. At the same time the rates of excretion of water and chloride reached their maximum values and in the subsequent periods they changed inversely with the rising plasma protein concentration. The inulin clearance did not change in the first 10 min. following the injection, then it started to increase slightly. The diodrast clearance followed almost

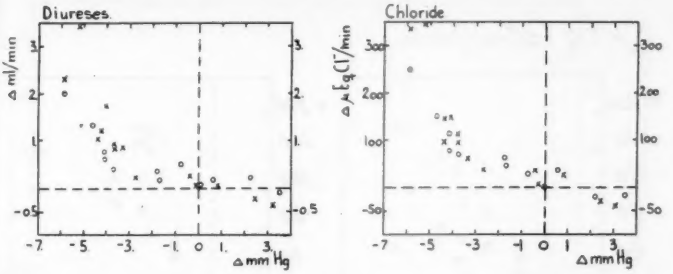


Fig. 2. The relationship between the change in C. O. P. of plasma and the rates of excess excretion of water (a) and chloride (b). × are data from dog T and ○ the data from dog S.

parallel with the inulin clearance throughout the experiment and thus the filtration fraction remained almost constant.

When later on the protein concentration approached the initial level the rates of excretion of chloride and water still remained above the initial values and sometimes were subject to a secondary increase. In order to evaluate to what extent these changes were caused by the increase in blood volume, other experiments were carried out in which a colloid was added to the salt solution injected. The results of such an experiment is presented as fig. 1 B. It is seen that in contrast to the dilution experiments no immediate increase occurred in diuresis and chloruresis, also there was no immediate change in inulin and diodrast clearances although the plasma volume increased more than in the experiment presented in fig. 1 A. Later on the clearances and also the diuresis and chloruresis increased.

Excretion of salt and water: From the experiment shown in fig. 1 A it is obvious that the changes in rates of excretion of salt and water are only inversely related to the change in protein concentration for a short period after the injection (15 to 20 minutes). Consequently only data from the first periods completed within this time interval were included in the diagrams in fig. 2, which show the relationships between changes in rate of excretion of water and chloride and the absolute changes in C. O. P. The rate of chloride excretion in the control periods of the experiments ranged from 3 to 100 μEq/min. Fig. 2 shows that within this wide range a certain absolute change in C. O. P. of the plasma caused the same absolute change in the rate of excretion of water and chloride. Furthermore it appears from fig. 2 that a proportionality

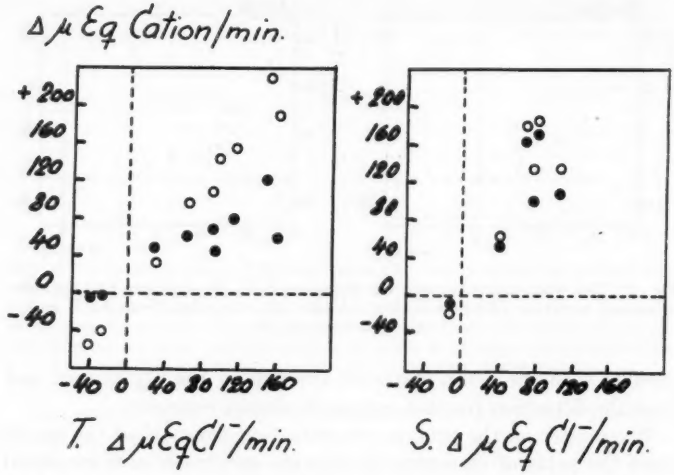


Fig. 3. The absolute changes in rates of excretion of chloride related to the absolute changes in rates of excretion of sodium (●) and sodium + potassium (○) for the dog T (a) and S (b).

was observed between the change in C. O. P. and the changes in the rates of excretion of chloride and water within a range of change in C. O. P. — 4.5 to 3 mm Hg. However, when the C. O. P. was lowered by more than 4.5 mm Hg the rates of excretion increased sharply. Finally the two diagrams in fig. 2 indicate that the concentration of chloride in the water excreted in excess over the control level in response to a reduction in C. O. P. of plasma was about the same as in a plasma ultrafiltrate *i. e.* about 100 μEq/ml.

From these results it is evident that the C. O. P. is a limiting factor for the rate of excretion of salt and water in dogs when there is any excretion at all of sodium and chloride.

(Only the data from a single exceptional experiment are not included in fig. 2. In this case the initial rates of water and electrolyte excretion were extremely low (chloride 0.5 μEq/min., potassium 20 μEq/min. and sodium zero) and a decrease of 3 mm Hg in C. O. P. of plasma only evoked an increase in the rate of chloride and sodium excretion of 4 μEq/min. and no detectable increases in potassium and water excretion. Probably, the salt reabsorbing mechanism of the tubules were not saturated at the beginning of this experiment.)

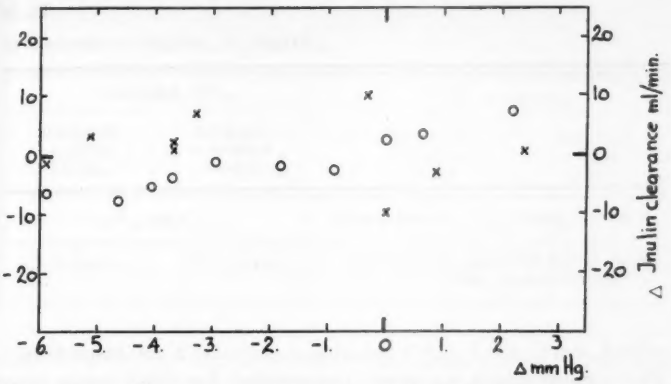


Fig. 4. The relationship between the changes in C. O. P. of the plasma and the change in inulin clearance from the second control period following the injection. (×) are data from dog T and (○) are data from dog S.

Cation excretion: Fig. 3 shows the relation between the rates of excess excretion of chloride and of sodium and total cation (sodium + potassium) in the first post-injection period. The discrepancies between the excess excretion of these cations and the excess excretion of chloride may be accounted for by changes in excretion of ammonia and bicarbonate. It will be seen that in dilution diuresis, not only is there an increase in the rates of excretion of sodium and chloride, but also in that of potassium and that in some cases the potassium excretion increased even more than the sodium excretion. This occurred especially in cases where the sodium excretion was low in proportion to the potassium excretion in the control periods.

The glomerular filtration rate: Fig. 4 shows the relationship between the change in C. O. P. and the difference between the inulin clearance values observed in the last control period and in the first post-injection period, which in all cases was completed within 15 minutes following the beginning of the injection. It will be seen that considering the experimental error involved in clearance determinations there was no measurable change in the filtration rate when the C. O. P. of plasma varied over a range of 8.5 mm Hg. The data presented are the clearances corrected for the dead space error by means of the procedure described in the previous publication (Bojesen 1954). The increase in inulin clearance following injection of the fluid with and without the

Table

Changes in plasma concentrations

	Cl^- mEq/Liter	
	Injection without a colloid	Injection with a colloid
2 control period — 1. control period	$+ 0.68 \pm 0.53$	
First period following the injection — second control period	0.13 ± 0.64	$- 0.40 \pm 1.1$

colloid were $+ 0.7 \pm 6.2$ ml and $- 1.3 \pm 5.2$ ml respectively. The corresponding increases, uncorrected for dead space error were $+ 4.5 \pm 7.4$ and $- 0.1 \pm 4.9$ ml. The relative modest differences between these corrected and uncorrected figures is a reflection of the fact that the dead space correction of the first post-injection period only amounted to 6 per cent of the inulin clearance values on an average. On the basis of previous experiments it may be estimated that the error on this correction hardly exceeded 30 per cent (cf. fig. 6 d BOJESEN 1954 which is based upon data from experiments on the same two dogs employed in this study). As a consequence, the uncertainty of the dead space correction should in average amount to some 2 per cent of the inulin clearance values. This figure is considerably lower than the standard deviation on the difference between the inulin clearance values obtained in the first and second control periods (under conditions of fairly constant rate of urine flow) viz. 3.6 ml. Consequently it was in conformity with expectancy that only a slightly larger standard deviation was found on the difference between inulin clearance values obtained in the last control and the first post-injection period.

Filtration fraction: The diodrast clearance was measured in 10 experiments where the injected fluid contained no colloid and 3 experiments where the injected fluid was isoncotic or hyperoncotic. The filtration fraction of plasma was calculated as the ratio inulin clearance/diodrast clearance and the average difference between this ratio in the first period following the injection and that of the last control period was 0.018 when fluid without a colloid was injected and 0.024 when fluid with colloid was employed. The average level of filtration fraction was 0.30. The

1.

of chloride, sodium and potassium.

Na^+ mEq/Liter		K^+ mEq/Liter	
Injection without a colloid	Injection with a colloid	Injection without a colloid	Injection with a colloid
$+ 0.03 \pm 0.5$		$- 0.15 \pm 0.18$	
$- 0.55 \pm 0.63$	$- 0.11 \pm 0.76$	$- 0.11 \pm 0.14$	0.00 ± 0.26

reproducibility of the filtration fraction from period to period is indicated by the standard deviation of the difference between the second and the first of the control periods. The difference was $+ 0.007 \pm 0.016$. From this standard deviation it may be calculated that the filtration fraction did not change significantly after the injection in the two groups of experiments.

Electrolyte concentrations in plasma: MAGNUS (1900) considered the possibility that the diuresis following injection of 0.9 per cent saline might actually be a "salt diuresis", viz. if following the injection water left the plasma more rapidly than "salt" leading to an increase in the plasma concentration of the latter. However, he declined this idea because 0.6 per cent sodium chloride gave as fast a diuretic response as did 0.9 per cent. In the present experiments the plasma electrolyte concentrations were followed. As shown in table 1 the changes are not statistically significant. Only the decrease in concentration of sodium after injection of colloid-free fluid was nearly significant (95 per cent chance).

As the plasma concentrations of cations have not changed, the assumption was made that with the changes in C. O. P. no material changes occurred in the concentrations of these ions in the ultrafiltrate since in the case of cations the effect of increases in the water phase of plasma will be counterbalanced by a decrease in Donnan effect. (Argumentation on this line is hardly warranted in the case of anions, since there is a good evidence for the occurrence of bound chloride in plasma (SCATCHARD and BLOCK 1949, KARUSH 1950).)

Arterial blood pressure: Immediately following the injection the arterial blood pressure usually increased some 5 mm Hg from a previous level of 130—140 mm Hg. This slight increase levelled

off during the first clearance period. This pattern was the same following injection of both types of solutions, and consequently the differences in excretion of salt and water were not due to differences in arterial blood pressure.

Discussion.

Among the observations made in the present experiments, the following two must be considered the most important. 1) the constancy of the filtration rate in spite of large changes in C. O. P. The filtration rate was not measurably influenced by changes in C. O. P. and did only change when the renal plasma flow changed. 2) the fact that the rate of excess excretion of cations and chloride were inversely related to the absolute change in C. O. P. and independent of the pre-existing rates of excretion, when these were above zero. It is interesting that these observations are in close agreement with the experimental findings of previous investigations dealing with the glomerular filtration rate under conditions of mechanically evoked diuresis or antidiuresis.

The first study dealing with the filtration rate in dilution diuresis is that of EGGLETON et al. (1940 b) in which creatinine clearance was determined in experiments on the isolated dog kidney and on anaesthetized dogs. Their results in anaesthetized dogs were similar to the results observed in the first post-injection periods of the experiments presented here, but in the isolated kidney they found an increase in creatinine clearance when plasma protein concentration was lowered. It may however be questioned whether the observed increase in creatinine clearance in the isolated kidney was due to an actual increase in filtration rate since SHANNON and WINTON (1940), found that at low diuresis creatinine clearance was about 30 per cent lower than inulin clearance whereas they were almost identical at relative high diuresis. WESSON et al. (1950) injected intravenously a large amount of isotonic saline in a few minutes. They did not measure the clearances during the initial 20 to 30 minutes following the injection. When they started clearance determinations they found that the filtration rate had increased; however the renal blood flow increased proportionally. WELT and ORLOFF (1951) and GOODEYER et al. (1949) observed that when the C. O. P. is increased by injection of albumin the salt and water excretion decreased without any detectable decrease

in the filtration rate. Since an antidiuretic response has also been evoked by injection of albumin in one renal artery (GOODEYER and GLENN 1952) it would appear that the antidiuretic effect is exerted directly on the kidney. The authors were uncertain as to the mechanism of the observed antidiuretic response, but most inclined to attribute it to a decrease in filtration rate; however they admitted that their clearance determinations were not too accurate.

Experiments with the other type of mechanical diuresis, the pressure diuresis, have shown that as long as the renal blood flow remained fairly constant changes in renal arterial pressure had no measurable influence on the filtration rate in dogs (SHIPLEY and STUDY 1951, MUELLER et al. 1951, and SELKURT 1951) and man (EPSTEIN et al. 1951). Nevertheless the excretion of salt and water varied considerably and parallel to the renal arterial pressure. In other experiments (SELKURT et al. 1949 and PITTS and DUNCAN 1950) in which the resistance in the renal artery of aorta was increased enough to produce a diminution in renal blood flow, it was found that not only was there a decrease in the rate of excretion of water and salt but also in the filtration rate. Thus the two observations stressed above, have also been made in previous studies on mechanical diureses. The interrelationship between the renal blood flow and filtration rate found in some earlier experiments was also observed in the present study whenever the filtration rate changed considerably.

Due to this agreement it was considered justifiable to draw certain conclusions which are incompatible with some generally accepted and fundamental hypotheses.

Most surprising to the author was the first mentioned result viz. the constancy of the glomerular filtration rate in spite of the large changes in C. O. P. because — as the following calculations will show — the accuracy of the clearance determinations is sufficient to disclose such changes in filtration rate that were to be expected if the concept (SMITH 1951) of a constant pressure in the capsular space was correct. With an average C. O. P. of 20 mm Hg (275 mm water) in the plasma entering the capillaries of the glomeruli and with a filtration fraction of 0.30, 30 mm Hg will be the C. O. P. in the plasma leaving the glomeruli. The average C. O. P. in the glomerular capillaries therefore is 25 mm Hg in the control periods. For the group of experiments in which the injected fluid had a C. O. P. above that of the plasma the calculated

C. O. P. in the *glomerular capillaries* increased some 2.0 mm Hg on an average, and in another group of experiments in which the injected fluid contained no colloid an average decrease of 5.8 mm Hg was obtained. The difference between these two groups of experiments in C. O. P. and thus in filtration pressure in the glomerular capillaries consequently has been about 8 mm Hg. Now by applying "Student's" t-test to the inulin clearances obtained in the two groups it has been calculated that there is only a probability of 0.01 that their true average values differ by more than 6.5 ml or 10 per cent of the average clearance value. From this figure and granted the filtration rate is proportional to the effective filtration pressure[1] across the glomerular membrane, one would have to conclude, that if Smith's assumption of a constant capsular pressure held true, an effective filtration pressure of at least 80 mm Hg should have existed across the glomerular membrane in the two dogs, $\left(\frac{8}{\text{effective filtration pressure}} \leq \frac{10}{100}\right)$. This is obviously not the case and consequently the capsular pressure must have changed. In this consideration the following assumptions have been made: 1) the permeability of the glomerular membrane remained constant, 2) the hydrostatic pressure in the capillaries was either constant or changed to the same extent following injection of the two types of fluid. The lack of change in filtration rate might be explained by assuming that the membrane is more dense when plasma proteins are diluted or by assuming that the vasa afferentia and efferentia have adjusted the hydrostatic pressure in the capillaries in such a way that the filtration pressure remains constant. Such assumptions, however make it difficult to find an explanation for the demonstrated relationship between C. O. P. and the excretion rates of salt and water. Moreover the renal blood flow was essentially unchanged whether the injected fluid contained colloid or not; this fact speaks against an adjustment of the filtration pressure in the capillaries by means of the vasa afferentia and efferentia. Thus, it seems most natural to reject the idea of constant pressure in Bowman's space and to assume that this pressure has changed closely parallel to the effective filtration pressure in the capillaries. According to this concept measurable changes in the filtration rate are brought about by

[1] Filtration pressure in the capillaries — capsular pressure.

changes in the tubular reabsorption. If this is accepted the following reasoning must be justifiable. The constant filtration rates observed in the first periods after the C. O. P. was changed indicate, that no changes have occurred in the fluid ("saline") reabsorption in these periods in spite of considerable changes in the capsular pressure and in intratubular pressure which must vary with the capsular pressure. As this pressure must be considered the "load" when dealing with the fluid ("saline") reabsorption the reabsorption of the bulk of the filtrate consequently is independent of the load and therefore has the same quality as reabsorption of glucose when its load exceeds the glucose Tm.

However, the experiments seem to indicate that also the reabsorption of the smaller fraction of the filtrate which occurs in the distal tubules is of the Tm type. If this is the case and the resistance to flow through the tubules is constant within a certain range of the capsular pressure then one should expect proportionality between the rate of fluid excretion and changes in this pressure. As may be seen from fig. 2 this seems to be the case for excretion rates of salt and water at changes in C. O. P. between -4.5 and $+3$ mm Hg. Furthermore a certain increase in this pressure should cause an absolute increase in excretion rate independent of the previous level of flow through the tubules if the absolute rate of reabsorption remains constant. This was actually found to hold true for pre-existing rates of excretion of chloride between 3—100 μEq/min. and for a corresponding range of pre-existing rates of water excretion. Finally a third detail of the results seems to render some support to the concept that saline reabsorption throughout the tubules is completely independent of the load. When the diagrams in fig. 2 are compared it will be seen that the ratio between chloride and water excreted in excess after lowering of the C. O. P. is about 100 μEq. per ml water *i. e.* a ratio corresponding to the ratio between chloride and water in plasma. The only detail in the results that may be claimed not to fit without further comment into the concept outlined is that the ratio between the excess excretion of sodium and potassium is very different from the ratio of the concentrations of these two cations in plasma. In most experiments high potassium/sodium ratio in the control periods are also followed by high potassium/sodium ratio during the dilution diuresis.

The simplest and for that reason most probable explanation of the experimental findings is that lowering of the C. O. P. causes

a slight (unmeasurable) increase in the filtration rate, whereas the reabsorption of salt and water remains absolutely uninfluenced by changes in the water and salt loads. The observations thus indicate that salt is a Tm substance throughout the tubules. The somewhat ambiguous expression "salt" Tm seems to the author preferable to more specific terms because at present we do not have any quite convincing evidence that the reabsorption of the bulk of the ions of the filtrate can be attributed to a reabsorption process specific for one particular ion.

The question may be raised why the concept of the reabsorption process of salt as a Tm has not been accepted at an earlier date in spite of the fact that it has been widely recognized that the tubular reabsorption of salt (Na^+) is an active process requiring cellular energy just as is the reabsorption of Tm limited substances such as glucose (WESSON and ANSLOW 1948, KRUHØFFER 1950 and many older experiments with cellular poisons). Furthermore, it has been observed by several investigators that the rate of salt (Na^+) reabsorption remained strikingly constant when the filtered load of salt (Na^+) was greatly changed by varying the plasma concentrations of sodium and chloride at constant filtration rates (SELKURT and POST 1950, LEVY and ANKERY 1952 and KRUHØFFER 1950). In spite of these data the idea of a "salt" Tm process for the salt reabsorption throughout the tubules has been repeatedly rejected because in all cases where it was intended to bring about changes in the filtered load of salt by varying the filtration rate it has been observed that the rate of reabsorption of salt (Na^+) *varied* fairly proportional to the filtration rate. However, previous investigations have not realized that the filtration rate is actually almost exclusively determined by the rate of tubular reabsorption of fluid and thus cannot be varied to a measurable extent independently of the latter. Consequently they did not consider the possibility that the changes observed in the filtration rate might merely be caused by changes in the rate of reabsorption of fluid. According to the concept here discussed this actually is the case and the most important factor responsible for the changes in the rate of fluid reabsorption and thus in the filtration rate, probably has been changes simultaneously occurring in the rate of renal blood flow.

In general it may be stated that according to the view discussed measurements of the filtration rate (inulin clearance etc.) do not provide information as to the size of the filtration pressure in the

capillaries and as to the resistance in the afferent and efferent vessels of the glomeruli. The size of the filtration rate is determined by the fluid reabsorbing capacity of the tubules and merely measures what may be termed "the intrarenal turn-over rate of saline". It seems then reasonable to consider the filtration fraction a measure of the stimulating effect on fluid reabsorption in the tubular cells excreted by each unit of blood traversing the kidney. At present we are lacking knowledge about the nature of the blood factor(s) responsible for this stimulation, however it does not seem to be simply a matter of oxygen supply. Before closing this discussion it must be emphasized that according to the view presented it is not possible — as generally accepted — to answer the question whether changes in the rate of excretion of water and the predominant electrolytes are caused by changes in the capsular pressure (glomerular factors) or by changes in tubular factors. This is an implication of the concept that a hydrostatic pressure gradient along the tubules is the only factor responsible for the propulsion of fluid through the tubules. Assuming a constant filtration pressure in the glomerular capillaries and constant gross permeability of the membrane, the rate of excretion of water is then determined by the hydrostatic gradient between the glomerular capillaries and the most distal site of water reabsorption and this again is determined not only by the rate of the tubular reabsorption but also by the distribution of the reabsorption along the tubules and the flow resistance. Therefore, even at a perfectly constant overall rate of reabsorption of fluid changes in the rates of excretion of salt and water may be caused not only by changes in capsular pressure (as in all probability in the present study) but also by two tubular factors viz. a shift in the distribution of the quantitatively constant overall rate of fluid reabsorption between the proximal and the distal reabsorption and by changes in the diameter of the lumen in the tubules. To this add the fact that the glomerular filtration rate can seldom be determined with an absolute accuracy larger than the changes in rate of excretion of water.

While abstaining from a further extension of this discussion, it may be pointed out that the above argumentation has implications as regards the mode of action of the antidiuretic hormone, the adrenocortical hormones and the renal control of the extracellular volume of the body.

Summary.

By using a technique that makes it possible to correct clearances for the "dead space" error induced by the upper urinary tract, the dilution diuresis phenomenon has been studied in series of experiments on two female unhydrated dogs. These studies showed an inverse relationship between the changes in C. O. P. of plasma and the absolute changes in the rate of excretion of chloride, water and cations (potassium + sodium). The absolute changes in salt excretion rate were rather independent of the initial level of excretion provided this exceeded a few microequivalents per min. However, the glomerular filtration rate was not affected to a measurable degree by a variation of some 8 mm Hg in the average C. O. P. in the glomerular capillaries.

These experimental results agree with those obtained by other authors in experiments dealing with mechanical diureses, dilution diuresis as well as pressure diuresis. Together with the results of many investigations on the rate of reabsorption of "salt" following changes in the concentration of "salt" in plasma with constant filtration rate, this seems to indicate that the rate of reabsorption of salt is just as independent of the "salt" load presented to the tubules as is the rate of reabsorption of glucose when the glucose load exceeds the glucose Tm. The rate of salt reabsorption, when salt is excreted, is consequently a "salt Tm". In the discussion it is shown that these observations imply that the inulin clearance is essentially a measure of the rate of tubular reabsorption of fluid.

The author wants to express his gratitude to Professor Einar Lundsgaard and Lektor Poul Kruhøffer for kind and extensive help with this work. I am also indebted to Dr. Svend Erik Nielsen for kind help with the determinations of colloid osmotic pressure.

References.

Adair, G. S., M. E. Adair and R. I. N. Greaves, J. Hyg. 1940. *40*. 548.

Berry, J. W., D. G. Chapell and R. B. Barnes, Industr. Engng. Chem. (Analyt.) 1946. *18*. 19.

Bojesen, E., Acta physiol. Scand. 1948. *16*. 1.

— Ibidem 1945. *32*. 39.

Cushny, A. R., The secretion of the Urine, London 1917.

EGGLETON, M. G., J. R. PAPPENHEIMER and F. R. WINTON, J. Physiol. 1940. *97*. 363.
— — — Ibidem. 1940. *98*. 336.
EPSTEIN, F. H., R. S. POST and M. MC DOWELL, Amer. J. Physiol. 1951. *167*. 781.
GOODEYER, A. V. N., E. R. PETERSON and A. S. RELMAN, J. Appl. Physiol. 1949. *1*. 671.
— and W. W. L. GLENN, Amer. J. Physiol. 1952. *168*. 66.
HOLM-JENSEN, I., Scandinav. J. Clin. and Lab. Invest. 1949. *1*. 70.
KARUSH, F., J. Amer. Chem. Soc. 1950. *72*. 2705.
KNOWLTON, F. P., J. Physiol. 1911. *43*. 219.
KRUHØFFER, P., Studies on Water-Electrolyte Excretion and Glomerular Activity in the Mammalian Kidney. Copenhagen 1950.
LEHMANN, J., Acta Paediat. 1939. *26*. 258.
LEVY, M. N. and J. L. ANKERY, Proc. Soc. Exp. Biol. N. Y. 1952. *79*. 491.
MAGNUS, R., Arch. exp. Path. Pharmak. 1900. *44*. 68.
— Ibidem 1901. *45*. 210.
MOUSTGAARD, J., Om proteinstoffernes Indflydelse paa Nyrefunktionen hos Hund. Copenhagen 1948. 64.
MUELLER, C. B., A. SURTSHIN, M. R. CARLIN and H. L. WHITE, Amer. J. Physiol. 1951. *165*. 411.
PHILIPS, R. A. and D. D. VAN SLYKE, J. Biol. Chem. 1950. *183*. 305.
PITTS, R. F. and DUNCAN, J. clin. Invest. 1950. *29*. 372.
REHBERG, P. B., Biochem. J. 1926. *20*. 497.
— Zbl. Inn. Med. 1929. *50*. 367.
RICHARDS, A. N., Proc. Roy. Soc. 1938. *126*, 398.
SCATCHARD, G. and E. S. BLACK, J. Phys. Colloid. Chem. 1949. *53*. 88.
SELKURT, E. E., Fed. Proc. 1951. *10*. 124.
— P. W. HALL and M. P. SPENCER, Amer. J. Physiol. 1949. *159*. 369.
— and R. S. POST, Ibidem 1950. *162*. 639.
SHANNON, J. A. and F. R. WINTON, J. Physiol. 1940. *98*. 97.
SHIPLEY, R. E. and R. S. STUDY, Amer. J. Physiol. 1951. *167*. 676.
SMITH, H. W., The kidney, Structure and Function in Health and Disease. New York 1951.
STARLING, E. H., J. Physiol. 1895. *19*. 312.
— The Fluids of the Body London 1909.
— and E. B. VERNEY, Proc. Roy. Soc. 1925. S. B. *97*. 321.
WALKER, A. M. and J. OLIVER, Amer. J. Physiol. 1941. *134*. 562.
— P. A. BOTT, J. OLIVER and M. C. MAC DOWELL, Ibidem 1941. *134*. 580.
WEARN, I. T. and A. N. RICHARDS, Ibidem 1924. *71*. 209.
WELT, L. G. and J. ORLOFF, J. clin. Invest. 1951. *30*. 751.
WESSON, L. G. and P. ANSLOW, Amer. J. Physiol. 1948. *153*. 465.
— — L. G. REISS, A. A. BOLOMEY and M. LADD, Ibidem 1950. *162*. 677.
WINTON, F. R., Physiol. Rev. 1937. *17*. 408.

From the Department of Physiology, University of Aarhus, Denmark.

The Absorption of Sodium Chloride in the Colon and the Distal Part of the Small Intestine.

By

SV. E. BUDOLFSEN.

Received 23 April 1954.

The first experiments on the absorption of sodium chloride in the small intestine were made by HEIDENHAIN in 1894, who observed, that with increasing infusion the absorption of sodium chloride in gram increased while the percentual absorption decreased. This result has later been confirmed by RABINOWITCH (1927), VISSCHER and DENNIS (1940/41) and VISSCHER et. al. (1944 b).

An absorption of sodium chloride against a concentration gradient was also observed by HEIDENHAIN (1894) in the small intestine of dogs, but in his experiment the solution of sodium chloride infused became concentrated during the absorption, however, without reaching the blood concentration of sodium chloride. A more significant absorption of sodium chloride against a concentration gradient without an increasing concentration during the absorption was observed by KATZENELLENBOGEN (1906), who reduced the water absorption in the intestine by adding a slowly absorbed substance to the solution infused. This result has later been confirmed by COBET (1913), BURNS and VISSCHER (1934), VISSCHER, ROEPKE and LIFSON (1945) and LIFSON (1940).

Experiments.

My experiments have been made on white rats. A colonic and an ileal segment were isolated immediately before the absorption experiment. The colon was isolated from the ileocoecal junction to the junction between the sigmoid and the rectum, while the

ileal segment was the distal 20 cm of the small intestine. The isolated segments were cleaned with an isotonic solution of glucose, the residues of which were removed by insufflating air through the segment. One hour later, while the animals were still in urethane anaesthesia (1.2—1.4 mg/g), the absorption experiments were made simultaneously in the two segments. The solution was infused through glass cannulae fixed to the ends of the isolated intestinal segments.

When the absorption had taken place, the animals were killed, the intestinal segments prepared free, and the intestinal content analysed. The absorption was determined as the difference between the infused and the remaining amount, *i. e.* the net absorption.

The determination of chloride was made according to the method of REHBERG (1926) with SCHNOHR's (1934) modification. The determination of sodium has been made by the method of OTTOSEN (1945).

The influence of the amount infused on the net-absorbed amounts of sodium and chloride is shown in table I. The total amount infused is varied by the concentration of the solutions infused. The absorption time is 60 minutes, and the volume infused 2 ml in all experiments.

Table I shows that the absorbed amount of sodium as well as chloride increases almost straight-lined with the infusion up to 1.25 per cent sodium chloride in the solution infused. When still more concentrated solutions are infused the net-absorption decreases except for chloride in the colonic segment, where the increase, however, is smaller than expected if the straight-lined increase had to be maintained. The percentual net-absorption of sodium as well as chloride in the two intestinal segments is practically a constant up to 1.95 per cent NaCl in the infused solution. An exception makes the percentual sodium net-absorption in the colon, which is lower by 0.25 than by 0.55 per cent NaCl; this difference is, however, statistically insignificant. The decrease in the percentual net-absorption from solutions of 1.95 per cent NaCl compared with solutions of lower concentrations is statistically significant.

As observed by COHNHEIM (1898 and 1899), HÖBER (1899), COBET (1913), GOLDSCHMIDT and DAYTON (1919-b), KNAFFL-LENZ and NOGAKI (1925), RABINOWITCH (1937), BURNS and VISSCHER (1934), MAC DOUGALL and VERZAR (1935), DENNIS (1940), VISSCHER et al. (1944-a) and VISSCHER and ROEPKE (1945) a transport of sodium as well as chloride from the intestinal epithelium into the intestinal content is going on during the absorption. As a consequence of this it is the net-absorption of the ions which is determined in

Table I.

Table I shows the absorption in the colon and in the distal part of the small intestine, when the concentration of sodium chloride in the solutions infused is varied. Absorption time 60 minutes. Volume infused 2 ml.

Infused solution			Colon				Small intestine			
			Sodium		Chloride		Sodium		Chloride	
Weight in g	% Chloride	% Sodium	Absorbed pr. 100 g animal	Absorbed in %	Absorbed pr. 100 g animal	Absorbed in %	Absorbed pr. 100 g animal	Absorbed in %	Absorbed pr. 100 g animal	Absorbed in %
173	0.15	0.10			1.00	44	0.63	56	1.27	73
162	—	—	0.60	50	1.47	79	1.00	83	1.65	89
186	—	—	0.61	55	1.18	72	0.88	77	1.36	83
143	—	—	0.49	36	1.50	71			1.19	57
165	—	—	0.23	18	0.79	43	0.56	43	1.15	61
198	—	—	0.12	11	0.59	38	0.70	64	1.09	71
Average			0.41	34	1.09	58	0.75	65	1.29	72
195	0.33	0.22	0.84	37	2.10	60	1.58	68	2.40	71
171	—	—	1.20	47	2.50	64	1.67	65	3.00	77
185	—	—	0.40	17	1.20	33	1.77	74	3.00	83
216	—	—	0.66	33	2.10	67	1.04	52	2.50	81
216	—	—	1.20	59	1.80	57	1.67	83	2.00	65
220	—	—	1.37	69	2.30	75	1.25	64	2.00	65
Average			0.95	44	2.00	59	1.50	68	2.48	74
203	0.55	0.35	1.70	48	3.35	63	2.20	63	3.55	66
168	—	—	1.50	35	3.87	60	3.00	71	4.86	75
203	—	—	1.60	43	3.27	61	2.70	77	4.34	81
164	—	—	1.40	29	3.22	46	2.42	51	3.81	54
164	—	—	2.36	50	4.62	66	3.21	67	4.47	64
153	—	—	2.55	50	4.69	67	3.74	73	4.71	63
Average			1.85	42	3.84	60	2.88	67	4.29	67
160	0.76	0.49	2.91	47	5.81	60	4.16	67	7.19	74
160	—	—	3.09	50	5.19	54	4.06	65	6.81	70
161	—	—	3.05	49	6.52	68	3.95	64	6.71	70
247	—	—	2.69	62	4.40	70	2.80	65	4.51	72
225	—	—	2.37	50	4.31	62	1.71	36	3.21	47
155	—	—	1.75	25	4.68	47	3.86	56	6.76	67
Average			2.64	47	5.15	60	3.42	59	5.86	67
253	1.18	0.77	2.32	37	5.00	52	2.13	34	4.81	50
164	—	—	2.43	25	6.94	47	4.04	43	7.74	53
225	—	—	1.56	22	4.72	44	1.61	23	3.71	35
180	—	—	2.25	26	5.72	43	1.57	18	4.67	35
204	—	—	2.25	30	6.50	55	3.30	43	6.44	54
Average			2.16	28	5.78	48	2.53	32	5.47	45

this work, and not the total-absorption. A determination of the intensity of the process: intestinal epithelium — intestinal content is difficult to make. VISSCHER et al. (1944-a and b) have tried to estimate this process by using radioactive isotopes of sodium, chloride and deuterium infused into the blood. In order to obtain the correct results it is, however, necessary that the isotopes applied do not injure the intestinal mucosa, and that the isotopes transported from the intestinal epithelium into the intestinal content are not reabsorbed from the intestinal content into the intestinal epithelium during the experiments. This latter claim is difficult to meet.

Briefly the conclusion drawn on the basis of former observations on the process: intestinal epithelium — intestinal content, is that while only a few results speak in favour of the process being a diffusion process there are plenty of results which speak in favour of the process being an active one, viz. a secretion.

The experiments in table II have been made with the purpose of getting some information about the process: intestinal epithelium — intestinal content. Especially it has been tried to find out whether it was possible to fix a blank for sodium as well as chloride. The experiments have been made as the aforementioned. Sodium chloride free solutions have been infused, viz. isotonic solutions of glucose and mannite and distilled water. Mannite is not absorbed in the small intestine of rats (HÖBER and HÖBER 1937), while glucose is easily absorbed in the small intestine, but difficultly in the colon of rats (DAVIDSON and GARRY 1939, BUDOLFSEN 1952).

In the *colon* the blanks vary between 0.45 and 0.83 mg sodium and 0.48 and 0.94 mg chloride per 100 g animal after 60 minutes absorption when the aforementioned solutions are used. At the end of the experiments the concentration of sodium in the intestinal content has been found to be 0.06—0.11 per cent, and of chloride to be 0.06—0.12 per cent. The highest concentrations were found when distilled water was infused. At the end of the experiments the volume of the intestinal content determined on the basis of the end-concentrations and the total amounts was found to be smaller when distilled water was infused than when the isotonic solutions were used.

In the *ileal segment* the blanks vary between 0.51 and 0.75 mg sodium and 0.56 and 0.97 mg chloride per 100 g animal when the aforementioned solutions were used. The end-concentra-

Table II.

Table II shows the blinds for various infused solutions — the volume infused in all experiments 2 ml — the absorption time varying.

Weight in g	Solutions infused	Colon				Small intestine				Absorption time in minutes
		Sodium		Chloride		Sodium		Chloride		
		mg pr. 100 g body weight	Conc. at the end in %	mg pr. 100 g body weight	Conc. at the end in %	mg pr. 100 g body weight	Conc. at the end in %	mg pr. 100 g body weight	Conc. at the end in %	
174	Isotonic	0.55	0.05	0.82	0.07	0.74	0.10	0.75	0.10	60
213	glucose	0.54	0.10	1.17	0.13	0.31	0.04	0.28	0.04	—
180		0.60	0.05	0.84	0.07	0.48	0.08	0.66	0.11	—
Average		0.57	0.07	0.94	0.09	0.51	0.07	0.56	0.08	60
172	Isotonic.....	0.76	0.05	0.76	0.05	0.88	0.08	0.83	0.08	60
224	mannite	0.83	0.07	0.76	0.07	0.58	0.07	0.78	0.08	—
197		0.89	0.06	0.91	0.06	0.77	0.08	0.91	0.09	—
Average		0.83	0.06	0.81	0.06	0.75	0.08	0.84	0.08	—
175	Distill.	0.49	0.12	0.43	0.11	0.92	0.40	1.19	0.52	60
221	water	0.33	0.10	0.38	0.12	0.43	0.32	0.51	0.38	—
178		0.54	0.12	0.63	0.14	0.81	0.29	1.22	0.43	—
Average		0.45	0.11	0.48	0.12	0.72	0.34	0.97	0.44	60
188	Isotonic		0.04		0.05	0.38	0.09	0.22	0.03	120
182	glucose	0.62	0.06	0.16	0.02	0.47	0.06	0.30	0.04	—
188		0.20	0.02	0.16	0.02	0.27	0.08	0.06	0.02	—
Average		0.41	0.04	0.16	0.03	0.37	0.08	0.19	0.03	120

tions fall in two distinct groups viz. for sodium 0.07—0.08 per cent in isotonic solutions and 0.34 per cent in distilled water and for chloride 0.08—0.08 per cent and 0.44 per cent. At the end of the experiments the relation between the intestinal volumes and the solutions infused is the same as in the colon.

The conclusion is that any relation between the volumes at the end of the experiments and the blanks cannot be demonstrated. The blank for sodium as well as chloride tends to be a constant, independent of the solutions infused.

In a few experiments in table II the absorption time was 120 minutes. This has only been possible in experiments with isotonic solutions of glucose and mannite. These experiments were made

with isotonic glucose solutions. The prolongation caused decreasing blanks in the colon as well as the ileum, especially for chloride.

These experiments thus confirm the results of several former investigations, viz. that during the absorption in the colon as well as the ileum there is a constant transport of both sodium and chloride ions from the intestinal epithelium to the intestinal content, and further that this transport can only partly be a diffusion process. The rather constant blank independent of the absorption rate and the osmotic concentration of the solutions infused favours the hypothesis that the process is a secretion. The constant blank does not necessitate an assumption of a constant transport of these ions, as the blank in experiments with this technic is fixed by two antagonistic processes: 1. from the intestinal epithelium to the intestinal content, 2. from the intestinal content to the intestinal epithelium. If these processes are varying in the same way the result will be a constant. The decreasing blanks when the absorption time is increased show, however, that the antagonistic processes do not always vary in the same way.

If we in the experiments in table I make a correction for the blanks, using the blanks for distilled water, we find that the percentual net-absorption of sodium as well as chloride is only slightly decreasing when the concentration of the solution infused increases from 0.25 to 1.25 per cent NaCl, while the fall becomes significantly greater by increasing the concentration from 1.25 to 1.95 per cent NaCl.

On an average the percentual chloride net-absorption is in both intestinal segments greater than the percentual sodium net-absorption. The difference is more pronounced in the colon which is shown by the quotient: $\frac{\text{chloride absorbed}}{\text{sodium absorbed}}$. This is in the colon, when results corrected for the blanks are used: 1.28—1.26—1.27—1.20—1.58, and in the ileal segment: 1.09—1.05—1.00—1.08—1.31 for solutions with 0.25—0.55—0.90—1.25 and 1.95 per cent NaCl. On an average the relation between the sodium and the chloride net-absorption in each intestinal segment seems to be rather constant up to 1.25 per cent NaCl. However, when studying the individual results in table I, results will be found in the colon as well as the ileal segment in which the percentual sodium net-absorption exceeds the percentual chloride net-absorption in the same intestinal segment.

Table III.

Table III shows the concentrations of sodium and chloride in the intestinal contents at the end of the experiments. Absorption time 60 minutes, volume infused 2 ml.

Solutions infused		Concentrations at the end			
		Colon		Small intestine	
% sodium	% chloride	% sodium	% chloride	% sodium	% chloride
0.10	0.15		0.09		
—	—	0.10	0.06		
—	—	0.06	0.06		
—	—	0.09	0.06		
—	—	0.10	0.10	0.22	0.21
—	—	0.13	0.13		
Average		0.10	0.08	0.22	0.21
0.22	0.33	0.10	0.15	0.29	0.41
—	—	0.08	0.09		
—	—	0.12	0.18		
—	—	0.14	0.13		
—	—	0.14	0.12	0.29	0.28
—	—	0.09	0.10	0.29	0.24
Average		0.11	0.13	0.29	0.31
0.36	0.54	0.22	0.22	0.31	0.41
—	—	0.20	0.19	0.24	0.27
—	—	0.18	0.19	0.25	0.29
—	—	0.23	0.26	0.27	0.37
—	—	0.20	0.22	0.21	0.34
—	—	0.18	0.21	0.27	0.38
Average		0.20	0.21	0.26	0.34
0.49	0.76	0.22	0.28	0.31	0.36
—	—	0.26	0.32	0.26	0.37
—	—	0.29	0.21	0.33	0.39
—	—	0.26	0.30	0.35	0.41
—	—	0.34	0.37	0.32	0.38
—	—	0.34	0.36	0.39	0.42
Average		0.29	0.31	0.33	0.39
0.77	1.18	0.31	0.40	0.39	0.46
—	—	0.45	0.49	0.40	0.50
—	—	0.41	0.45	0.46	0.60
—	—	0.40	0.47	0.49	0.60
—	—	0.29	0.29	0.40	0.49
Average		0.37	0.42	0.43	0.53

Simultaneously with the determinations of the net-absorption of sodium and chloride the concentration of the same ions have been determined by analyses of the intestinal content at the end of the experiments. These results can be seen in table III, which shows that in the *colon* the concentration decreased averagely for both ions independent of the concentration of the solution infused. In the *small intestine* the concentration for sodium as well as chloride decreased also in the iso- and hypertonic solutions during the absorption, while in hypotonic solutions it increases with the exception of chloride from a 0.55 per cent NaCl-solution where there is a small decrease. On account of the small volumes of the intestinal content there are, however, only a few determinations of the chloride and sodium concentration at the end of the experiments with 0.25 and 0.55 per cent NaCl.

If we express the intensity of the concentration variations after an absorption time of 60 minutes by the term:

$$\frac{\text{concentration at the end} \times 100}{\text{concentration at the infusion time}}$$

as seen in table IV we also here see the different variations in the two intestinal segments during absorption of hypotonic solutions, and the homogeneous variations during absorption of iso- and hypertonic solutions.

Table IV.

Table IV shows $\frac{\textit{concentration at the end} \times 100}{\textit{concentration at the infusion time}}$ *for sodium as well as chloride in the two intestinal segments after an absorption time of 60 minutes.*

Infused solutions.....		0.25	0.55	0.90	1.25	1.90 % NaCl
Colon	Sodium	95.0	51.1	55.7	51.8	48.3
	Chloride.....	55.5	39.0	39.2	35.3	36.3
Small Intestine	Sodium	221.0	134.1	72.3	50.2	55.8
	Chloride.....	143.8	95.2	63.5	49.1	44.8

Further we observe that the decrease in concentration in the colonic as well as the ileal segment is greater for chloride than for sodium, equivalently with the greater absorption of chloride than of sodium. Striking is the practically equal decrease in concentration in the various solutions of sodium chloride in colon with the exception of 0.25 per cent NaCl; the concentration in all solutions between 0.55 and 1.95 per cent NaCl falls to 35—40 per cent for

chloride and to about 50 per cent for sodium of the concentration of the solutions infused. In the small intestine the decrease in concentration seems to vary with the concentration of the solution infused for sodium as well as chloride, and to be greatest from the most concentrated solution. Only in the more concentrated solutions the intensity of the variation in concentration appears to be of the same intensity in the two intestinal segments.

At the end of the experiment the water net-absorption has been determined as the difference between the volume infused and the volume of the intestinal content; the latter was determined from the concentration and the total amount of substance at the end of the experiment.

In the *colon* the water net-absorption was found negative after 60 minutes absorption of 2 ml 0.55—0.90—1.25 and 1.95 per cent NaCl, the volume increased 0.08—0.10—0.26 and 1.08 ml. In the *ileal segment* the water net-absorption was positive when 2 ml 0.55—0.90 and 1.25 per cent NaCl were injected for 60 minutes; the absorbed volumes were 1.33—1.01 and 0.78 ml. Only after infusion of 2 ml 1.95 per cent NaCl in the ileal segment the water net-absorption was found negative, viz. an increase of 0.44 ml.

The difference observed in the intensity of the concentration variations for chloride and sodium in the two intestinal segments can thus be explained by a difference in the intensity of the water net-absorption.

The blood chloride concentration in 53 animals was averagely 0.328 per cent, while the sodium concentration was averagely 0.308 per cent in 38 animals.

During the absorption of sodium as well as chloride in the colon the concentration of sodium as well as chloride in the intestinal content falls below the concentration of both ions in the blood, except when 1.95 per cent NaCl is used. In the small intestine the hypotonic solution concentrates to about the blood concentration of these ions.

Discussion.

An increasing net-absorption of sodium chloride with increasing concentration of the solution infused (and total amount infused) has been observed by HEIDENHAIN (1894). RABINOWITCH (1927), VISSCHER and DENNIS (1940/41) and VISSCHER et al. (1944-b)

observed the same for chloride alone in dog's intestines, and MAC DOUGALL and VERZAR (1935) the same in the jejunum of rats.

RABINOWITCH (1927) observed in his experiments on dog small intestines — just as I have observed in rat experiments — that when the NaCl-concentration of the solutions infused increased too much the chloride net-absorption decreased. The limit was in his experiments 1.5 per cent NaCl. VISSCHER et al. (1944-b) did not observe such a limit, possibly on account of the great difference in concentration between the solutions tested (0.3—0.9 and 2.7 per cent NaCl).

The decrease in net-absorption for sodium as well as chloride when strongly hypertonic solutions are used must be caused by an effect on the intestinal mucosa by which either the absorption power decreases or the blank increases. The latter effect has formerly been observed by COHNHEIM (1899) when using strongly hypertonic glucose solutions, but it has not been confirmed by VISSCHER et al. (1944-b) in their experiments with radioactive isotopes on dogs. On the contrary, they observed that the chloride transport from the intestinal mucosa to the intestinal content was a constant, uninfluenced by the chloride concentration of the intestinal content. My experiments have not shown any relation between the osmotic concentration of the solution infused and the blank.

The relatively greater net-absorption of chloride than of sodium, such as it has been observed in the majority of my experiments has formerly been observed by INGRAHAM and VISSCHER (1937) in sodium chloride experiments with the small intestine of dogs, where it was shown that the decrease in concentration was greater for chloride than for sodium per time-unit, when an isotonic mixture of NaCl and $MgSO_4$ was used. These experiments, however, are complicated by the addition of $MgSO_4$.

Experiments of this kind have not been made with colon before.

In his experiments with sodium absorption through frog-skin USSING (1949-a) observed in most cases a greater sodium than chloride net-absorption, but a fixed relation did not exist.

The relation between the chloride and the sodium net-absorption appears to be different in the two intestinal segments: the chloride net-absorption compared with the sodium net-absorption being 20 per cent greater in the colon than in the ileal segment. The

most hypertonic solution (1.95 per cent NaCl) disturbs in the colon as well as in the ileum the relation between the percentual sodium and chloride net-absorption, the former decreasing in proportion to the latter. This may be a sign of a different absorption mechanism of the two ions, but may also be explained by a relatively greater increase of the sodium blank than of the chloride blank. An increase of the intestinal secretion as a result of the strongly hypertonic solutions is able to provoke this because the sodium concentration of the intestinal secretion is higher than the chloride concentration (DE BEER, JOHNSTON and WILSON 1935).

The variations of the concentrations in my experiments during the absorption of hypertonic NaCl solutions in the colon and the ileal segment agree with former experiments of HEIDENHAIN (1894), BURNS and VISSCHER (1934), VISSCHER et al. (1944), GOLDSCHMIDT and DAYTON (1919-b) and MAC DOUGALL and VERZAR (1935).

The results from isotonic solutions in my experiments also agree with those of the aforementioned investigators.

BURNS and VISSCHER (1934) concluded that the concentration decrease for chloride in the small intestine stops, when the chloride concentration of the blood is reached. Generally this does not agree with my results, where some experiments show a concentration decrease far below the chloride concentration of the blood. In GOLDSCHMIDT and DAYTON's (1919-a) experiments on dogs colon the decrease in chloride concentration, when hypertonic and isotonic solutions were absorbed, stopped at the blood concentration. In my colon experiments the decrease continues below the blood concentration of chloride.

The increase in concentration in the ileal segment during absorption of a hypotonic NaCl solution also agrees with the experiments of the aforementioned investigators. On the other hand, the uniform decrease in concentration in the colon during the absorption of a hypotonic solution does not agree with the results from colon of dogs found by GOLDSCHMIDT and DAYTON (1919-a), where the same variations as in the ileal segment were observed. The small increase in a few of my experiments with 0.1 per cent sodium may be caused by the sodium blank; in the intestinal secretion the sodium concentration is about 0.1 per cent.

This disagreement between GOLDSCHMIDT and DAYTON's (1919-a) results and mine may be caused by the different experimental time or the different experimental animals. The former

used an experimental time of 20 minutes, while my results have been obtained in experiments with an experimental time of 60 minutes. Some experiments show that the difference is not caused by the difference in experimental time, as a steady decrease in concentration for sodium as well as chloride is observed during the whole absorption period.

The variations in concentration during the absorption of pure sodium chloride solutions in the colon are identical with those observed in the small intestine, when a slowly absorbed substance is added to the solution infused — an observation first made by KATZENELLENBOGEN (1906). In order to exclude that residues of the glucose solution used for cleaning the intestinal segment may have been responsible for the concentration variations observed in the colon during absorption of a hypotonic NaCl-solution a series of experiments have been made in which distilled water was used for cleaning the intestinal segment instead of isotonic glucose solution. In these experiments the concentration variations are the same as in the experiments where a glucose solution is used for cleaning, so that it cannot have been residues of the glucose which have provoked the absorption of chloride and sodium against the concentration gradient in the colon.

Conferring table I and table III it will be observed that in the colon there is an extensive absorption of sodium as well as chloride against a concentration gradient from solutions of 0.25 and 0.55 per cent NaCl, because the concentration of sodium as well as chloride the whole time is below the concentration of the same ions in the blood. This can be explained only by accepting the presence of an active absorption process. At the end of the experiments on the ileal segment the volume of the intestinal content has most often been too small to allow a determination of the concentration. This was only possible in one experiment with 0.25 per cent NaCl, and at the end of this the concentration of chloride as well as sodium was found to be below the concentration of the same ions in the blood. When 0.55 per cent NaCl was infused it was possible to determine the concentration at the end of 3 out of 6 experiments, and in these the sodium concentration was a little below the blood sodium concentration, while the chloride concentration in the intestinal content was lower than the blood chloride concentration in 2 experiments, so that an absorption of sodium and chloride against the concentration gradient was less significant in the ileal segment of rats than in the colon.

Thus the experiments have with certainty shown an absorption of sodium as well as chloride against a concentration gradient in the colon of rats, and so demonstrated the presence of an active absorption mechanism for these ions. The observed almost constant percentual net-absorption of sodium chloride in solutions with 0.25 to 1.25 per cent NaCl strongly suggests that a passive absorption mechanism in form of diffusion is also important for the absorption of sodium chloride, and that this last mentioned factor is probably a limiting factor for the intensity of the absorption process, while we otherwise have to expect a decreasing percentual absorption with increasing infusion of sodium chloride.

The presence of an active absorption mechanism for salt solutions in the small intestine has formerly been supposed on the basis of the observations of KATZENELLENBOGEN (1906), COBET (1913), BURNS and VISSCHER (1934) and INGRAHAM and VISSCHER (1935/36), but these observations have only been possible when the water-net-absorption was restained artificially. KROGH (1946) has argued against the fluid circuit theory of INGRAHAM and VISSCHER (1937) especially on the account of the conditions artificially induced under which the experiments, upon which the theory was based, were carried out. He was of the opinion that in the intact organism the amount of difficultly absorbed substances was too small to provoke an absorption against the concentration gradient. This objection cannot be made in the case of the colon of white rats, where it is possible with no tricks to observe an absorption of sodium as well as chloride ions against a concentration gradient.

My observations are able to fit with both hypotheses of active salt absorption: the fluid circuit theory of INGRAHAM and VISSCHER (1937), and the theory of FRANCK and MAYER (1947), by USSING (1949-b) called the solute circuit system.

Summary.

The absorption of sodium and chloride ions in the colon and the distal part of the ileum of rats has been investigated.

The amounts absorbed of both ions were found to increase straight-lined with the concentration until a concentration higher than 1.25 per cent NaCl was reached; at a higher concentration (1.95 per cent) the net-absorption of both ions decreased. The percentual absorption after correction for a blank was only slightly

decreasing in both intestinal segments until a concentration higher than 1.25 per cent NaCl was reached, after which it decreased significantly.

A rather constant blank for sodium as well as chloride was observed in both intestinal segments when sodium chloride-free solutions were used, independent of the solutions infused.

During the absorption the sodium as well as the chloride concentration in the intestinal content decreased in the colon when hyper-, iso- and hypotonic solutions were infused; it decreased also in the ileal segment when hyper- and isotonic solutions were infused, but increased when hypotonic solutions were used.

Thus it was possible to observe an absorption of sodium as well as chloride against a concentration gradient in the colon. In the ileal segment it was possible to observe an absorption of both ions against a concentration gradient too in some experiments, because the concentration increase during the absorption of hypotonic solutions did not obtain such an intensity that the blood concentration of chloride and sodium were reached.

References.

Beer, E. J., C. G. Johnston and D. W. Wilson, J. Biol. Chem. 1935. *108*. 113.

Burns, H. S. and M. B. Visscher, Amer. J. Physiol. 1934. *110*. 490.

Budolfsen, S. E., Diss., Aarhus 1952.

Cobet, R., Pflüg. Arch. ges. Physiol. 1913. *150*. 325.

Cohnheim, O., Zeitschr. f. Biol. 1898. *36*. 129.

— ibidem 1899. *37*. 443.

Davidson, J. N. and R. C. Garry, J. Physiol. 1939. *96*. 172.

Dennis, C., Amer. J. Physiol. 1940. *129*. 171.

— and M. B. Visscher, ibidem 1940. *129*. 176.

— — ibidem 1940/41. *131*. 402.

Franck, J. and J. E. Mayer, Arch. Biochem. 1947. *297*. 14.

Goldschmidt, S. and A. B. Dayton, Amer. J. Physiol. 1919-a. *48*. 440.

— — ibidem 1919-b. *48*. 459.

Heidenhain, E., Pflüg. Arch. ges. Physiol. 1894. *56*. 579.

Höber, R., ibidem 1899. *74*. 246.

— and J. Höber, J. Cell. Comp. Physiol. 1937. *10*. 401.

Ingraham, R. C. and M. B. Visscher, Amer. J. Physiol. 1935/36. 114. 676.

— — ibidem 1937. *121*. 771.

Katzenellenbogen, M., Pflüg. Arch. ges. Physiol. 1906. *114*. 522.

Knaffl-Lenz, E. and S. Nogaki, Arch. exp. Path. Pharmak. 1925. *105*. 109.

KROGH, A., Proc. Roy. Soc. 1946. *133*. 140.
LIFSON, N., Amer. J. Physiol. 1940. *128*. 603.
MAC DOUGALL, E. J. and V. VERZAR, Pflüg. Arch. ges. Physiol. 1935. *226*. 321.
OTTOSEN, D., Diss. Aarhus 1945.
RABINOWITCH, J., Amer. J. Physiol. 1927. *82*. 279.
REHBERG, P. E., Biochem. J. 1926. *20*. 483.
SCHNOHR, E., Diss. Kbhvn 1934.
USSING, H. H., Acta physiol. Scand. 1949. *17*. 1.
— Physiol. Rev. 1949. *29*. 127.
VISSCHER, M. B., R. H. VARCO, C. W. CARR, R. B. DEAN and D. ERICSON, Amer. J. Physiol. 1944-a. *141*. 488.
VISSCHER, M. B., E. S. FETCHER, C. W. CARR, H. P. GREGOR, M. S. BUSHEY and D. E. BARBER, ibidem 1944-b. *142*. 550.
VISSCHER, M. B. and R. R. ROEPKE, ibidem 1945. *144*. 468.
— — and N. LIFSON, ibidem 1945. *144*. 457.

Histological Department, Karolinska Institutet, Stockholm, Sweden.

Influence of Cortisone Acetate on Thyroid Stimulation by Means of Thyrotropic Hormone in Young Guinea Pigs.

By

U. BODLUND and L. GYLLENSTEN.

Received 24 April 1954.

Introduction.

Judged from investigations on the turnover of radioiodine most authors report an inhibitory action on thyroid function by adrenal cortical steroids in rats or human beings (BEIERWALTES et al. 1950, HILL et al. 1950, MONEY et al. 1950, 1951, WOLFSON et al. 1950, PERRY 1951, VERZAR and VIDOVIC 1951, BERSON and YALOW 1952, FREDERICKSON et al. 1952, SHELLABERGER et al. 1953, and others). The statements are, however, not consistent. No influence of cortisone on the I^{131} turnover was found by ALBERT et al. (1952) and MIGEON et al. (1952) in rats and by D'ANGELO et al. (1953) in goitrous rats. INGBAR (1953) reported an increased clearance of iodide after cortisone administration in rats and proposed that the decreased thyroid accumulation of radioiodine after cortisone treatment was the mere consequence of the increased urinary excretion and the resultant lowering of plasma iodide.

Other criteria on thyroid function have also given divergent results as to the action of adrenal steroids on the thyroid. A slight lowering of B M R has been reported by HILL et al. (1950) and WOLFSON et al. (1950). The influence of the adrenal cortex on the basal metabolism may, however, at least in part, be due to a

direct effect not mediated via the thyroid (BEIERWALTES et al. 1950; studies on the effect of cortisone on basal oxygen consumption in patients with untreated myxedema). The rise in B M R, which follows upon treatment with thyroxine or thyrotropic hormone, has been reported to be counteracted by the administration of adrenal cortical extracts (OEHME 1936, HOEN et al. 1939). KOELSCHE and KENDALL (1935) have found a lowering of the increased nitrogen excretion and pulse rate, seen in experimental hyperthyroidism, by means of suprarenal cortical hormone. ABELIN and ALTHAUS (1942) described a similar antagonistic effect as regards glycogen metabolism of the liver. A decrease of the pituitary content of thyrotropic hormone after cortisone treatment in rats subjected to thyroidectomy was reported by LEDERER (1952). This was contrary to the experience of D'ANGELO et al. (1953) who found an increase of thyrotropic hormone in the blood and pituitary of goitrous rats similarly treated. HALMI (1952) found a slight enhancement of thyroid stimulation through thyrotropic hormone in hypophysectomized rats after cortisone administration, judged from average thyroid cell height. In contrast to this WOODBURY et al. (1951), VERZAR and VIDOVIC (1952) and EPSTEIN et al. (1953) found inhibition of thyroid stimulation by introducing thyrotropin in hypophysectomized rats after treatment with cortisone, judged from the uptake of radioiodine. HALMI and BARKER (1952) described histologic changes in the thyroid and hypophysis of rats, suggesting an enhanced output of thyrotropin through the administration of cortisone, but no measurements of acinar cell height and no statistical analysis were published. Histologic signs of thyroid stimulation by cortisone were also reported by HIGGINS et al. (1951) and SPIRTOS (1953), still without any quantitative analysis. Contrary to these reports, no histologic activation was found by WINTER et al. (1950), and decreased histologic activity by MERCIER et al. (1951).

Comprehensive reviews on the interactions of pituitary, thyroid and adrenal cortex are published by SELYE, and SELYE and HORAVA (1950, 1951, 1952, 1953).

It is a well known fact that thyroid histology and function exhibit great variations for example between different animal species and different breeding stocks of the same species, during the seasons of the year, between animals given different diets. These variations greatly influence the assays of thyroid function.

As regards the action of cortisone on thyroid function differences may exist between hypothyroid, euthyroid and hyperthyroid animals as indicated by some of the before mentioned authors (for instance D'ANGELO et al. 1953). When judging from the uptake of radioactive indicators, the possibility of a cortisone effect on diuresis must be kept in mind, as pointed out by INGBAR (1953).

Cortisone causes an involution of the thymus and lymphatic tissue, which means another complication for the estimation of the cortisone action on thyroid. RAWSON (1949, 1952) and RAWSON et al. (1942, 1943, 1949) have demonstrated that the thymus and lymphatic tissue *in vitro* inactivate thyrotropic hormone. GYLLENSTEN (1953) found a slight thyroid hyperactivity and an increased thyroid reaction to exogenous thyrotropic hormone in growing guinea pigs after subtotal thymectomy. Thus, it may be possible that the thymus and lymphatic tissue *in vivo* are capable of inactivating thyrotropic hormone. An involution of the said tissues after cortisone treatment could, then, perhaps cause an indirect stimulation of the thyroid, by means of a decreased inactivation of endogenous thyrotropic hormone in the decreased mass of the thymus and lymphatic tissue.

The aim of the present investigation is to study the effect of cortisone on thyroid histology and function and on the stimulation of thyroid by exogenous thyrotropic hormone, taking the reactions of the thymus and lymphatic tissue and the previous critical reflections into account.

Methods.

Young guinea pigs, weighing about 200—250 g, were grouped in pairs, according to sex and similarity of body weight, one animal in each pair serving as experimental animal injected with cortisone, the other as corresponding control. The statistical calculations of differences were consequently based on paired comparisons between concomitantly bred and prepared animals. The animals were fed *ad libitum* on hay, turnips and oats.

Among 19 pairs of guinea pigs one animal of every pair was injected with cortisone acetate (Upjohn Co.), 5 mg twice a day intraperitoneally for 10 days. The animals injected with cortisone and their corresponding controls were killed on the eleventh day after the beginning of the injections and 24 hours after the last injection.

Among 28 pairs of animals one animal of every pair was treated with cortisone acetate as previously mentioned. On the eighth, ninth and tenth day, all animals of these 28 pairs were injected subcutaneously with 1 MsE thyrotropic hormone (according to the definition by HEYL and LAQUEUR) dissolved in 0.5 ml Ringer's solution.

The thyrotropic hormone preparation was Ambinon (Pharmacia), a pituitary extract from cows, which also contains gonadotropic factors (the latter having no appreciable effect on the action of this extract on the thyroid, according to BORELL 1945).

One hour (LAMBERG 1953) before being killed, the guinea pigs were injected subcutaneously with 0.25 mC radiophosphate per kg body weight. The radiophosphate (orthophosphate, labelled with P^{32}, obtained from A. E. R. E., England) was dissolved in 0.9 % saline at pH 6.7 containing 0.01 % phosphate as carrier.

The animals were killed by a blow on the back of the skull. The thyroid glands were dissected, cleaned from surrounding tissue, weighed and immediately subjected to measuring by radioactivity. After that the thyroids were fixed in 10 % formaldehyde solution, embedded in paraffin and cut in sections 5 microns thick through the middle portion of the gland. The slides were stained in iron-alum-hematoxylin. The heights of 25 acinar cells from every animal were measured after projection on the focussing screen in a microcamera at a magnification of 1,000. The magnified cells were measured with a measuring stick having an exactitude of 0.5 mm. When measuring, it was not known how the animal had been treated, from which the actual slide had been prepared. The mean of the recorded heights on the 25 cells served as a criterion for the animal in question in further calculations.

The accumulation of P^{32} in the thyroid has been used as a test on thyroid activity and stimulation by BORELL and HOLMGREN (1949), BRIMBLECOMBE et al. (1952), BESFORD et al. (1952), LAMBERG (1953) and GYLLENSTEN and RINGERTZ (1954).

In the present investigation the radioactivity was measured with a T G C 2 (Tracerlab) Geiger-Müller tube and expressed in counts per minute and mg of tissue. According to calculations by GYLLENSTEN and RINGERTZ (1954) variations in self-absorption between pieces of tissue of different weights have no appreciable effect on the recorded activity, when working with such small specimens as the present.

Because of the influences of thyroid (and cortisone) on phosphorus metabolism and urinary excretion small pieces of fat, muscle, and liver tissue were dried in air and subjected to similar activity measurings as the thyroids, so that thyroid activity could be expressed in relation to activity of the said tissues.

Among 19 pairs of animals, used for determination of cortisone action without any exogenous thyrotropic stimulation, a quantitative preparation was made also of the cervical and inguinal lymph nodes (for dissection technique, see GYLLENSTEN 1953), thymus, spleen, and adrenals. Specimens of these organs were dried and the activities measured.

Results.

The mean values of thyroid weight, mean acinar cell height and radioactivity in counts per minute and mg of tissue are recorded in *Table 1.*

Table 1.

Mean values of thyroid weight, mean acinar cell height and P^{32} accumulation (counts per minute and mg of tissue) in (1) guinea pigs without hormone treatment (Group O), (2) guinea pigs treated with cortisone acetate (Group C), (3) guinea pigs treated with thyrotropic hormone (Group T), and (4) guinea pigs treated with cortisone acetate and thyrotropic hormone (Group CT). Groups O and C, and Groups T and CT consist of corresponding pairs of animals, respectively.

Group	(mg)	(mg/g body w.)	(Microns)	P^{32} activity (counts/min./mg)				Thyroid activity relative[1] to activity of			Number of animals
	Thyroid weight	Relative thyroid weight	Mean acinar cell height	Thyroid	Fat	Muscle	Liver	Fat	Muscle	Liver	
O	33.4	0.135	4.16	37.6	12.7	13.2	156.5	3.64	3.00	0.261	19
C	40.2	0.158	4.55	38.9	11.2	11.3	153.2	4.12	3.81	0.258	19
T	35.9	0.130	5.65	39.0	11.8	14.0	169.7	3.76	3.17	0.232	28
CT	41.4	0.152	5.63	37.8	14.8	16.6	144.0	3.13	2.41	0.238	28

As apparent from Table 1, the injections with cortisone acetate caused an increase of thyroid weight, mean acinar cell height and thyroid accumulation of P^{32}. When the cortisone treatment was followed by thyroid stimulation with exogenous thyrotropic hormone, however, no increase of mean acinar cell height or accumulation of labelled phosphate could be demonstrated in the guinea pigs subjected to cortisone injections as compared with control animals, only given injections with thyrotropic hormone. Contrary to this, the weight increase of the thyroid in the animals given cortisone injections was apparent also after stimulation by thyrotropic hormone.

The fact, that assays of thyrotropic influences vary very much when performed on groups of animals on different occasions, is apparent from Table 1 as regards the effect of thyrotropic hormone

[1] The mean relative activity of thyroid for a group of animals was calculated as the mean of individual relative thyroid activities. This is the reason that it does not correspond to the quotients of the means.

Table 2.

Mean percentage increase of thyroid function in guinea pigs subjected to cortisone injections (Group C) as compared with paired controls (Group O) and in guinea pigs subjected to cortisone treatment followed by injections with thyrotropic hormone (Group CT) as compared with paired controls, only given thyrotropic injections (Group T). Statistical conventions according to BONNIER *and* TEDIN *(1940): sign × means a p-value less than 0.05 and sign × × a p-value less than 0.01.*

	Thyroid weight	Relative thyroid weight	Mean acinar cell height	Thyroid uptake of P^{32}	Thyroid uptake of P^{32} relative to P^{32} uptake in		
					Fat	Muscle	Liver
Group C (increase in per cent) ...	21.26	19.68	10.42	3.80	29.26	32.11	1.95
t	3.199	2.836	2.462	0.817	2.277	2.780	0.387
sign	× ×	×	×	—	×	×	—
Group CT (increase in per cent) ...	19.57	23.82	0.29	0.79	— 3.43	— 14.79	3.21
t	3.216	3.300	0.136	0.161	— 0.363	— 1.879	0.714
sign	× ×	× ×	—	—	—	—	—
Difference in increase between Group C and Group CT	1.69	— 4.14	10.14	3.01	32.69	46.89	— 1.27
t	0.185	— 0.413	2.350	0.426	2.096	3.481	— 0.188
sign	—	—	×	—	×	× ×	—

on relative thyroid weight. Animals of Group T did not show any increase of relative thyroid weight, compared with the animals of Group O, though the former had been subjected to stimulation with thyrotropic hormone. Such variations between groups of animals constitute a reason to use paired comparisons on concomitantly treated animals.

Based on paired comparisons and t-analysis according to the Student's test the increase of thyroid weight after cortisone treatment proves to be statistically significant (p less than 0.01) both without and with a subsequent stimulation with thyrotropic hormone (Table 2). The increase of thyroid function, as judged from paired comparisons of mean acinar cell height and relative P^{32} accumulation, proves to be statistically almost significant (p less than 0.05) in animals given cortisone injections (Group C compared with Group O) but not when the cortisone treatment was

followed by stimulation with thyrotropic hormone (Group CT compared with Group T). As seen in Table 2, the difference in reaction between animals given cortisone injection and animals given cortisone injections and injections with thyrotropic hormone proves to be statistically significant or almost significant.

The uptake of P^{32} in thyroid relative to the uptake in liver tissue shows no increase in the animals subjected to cortisone injections, contrary to the uptake relative to fat or muscle tissue. This indicates an increase of liver activity, compared with fat or muscle tissue, and may probably depend on a cortisone stimulation of the turnover of phosphorus compounds in the liver, perhaps phospholipids (FRAENKEL-CONRAT and LI 1949, GESCHWIND et al. 1950, ALTMAN et al. 1951).

Among the guinea pigs given injections with cortisone acetate there was a statistically highly significant weight decrease of lymph node tissue and of thymus, as apparent from Table 3. After ten days of cortisone injections lymph node weight had decreased about 20—25 % and the thymus about 37 %. No significant change of body weight or of adrenal weight was demonstrable. No sexual differences were revealed.

Table 3.

Weight changes in guinea pigs injected with cortisone acetate in per cent of the figures for the corresponding control animals. Paired comparisons, 19 pairs.

	Body weight	Cervical lymph nodes	Inguinal lymph nodes	Spleen	Thymus	Adrenals
Mean increase of cortisone injected animals in per cent.	+ 1.89	— 22.74	— 24.58	+ 6.32	— 37.21	— 7.84
Standard error	1.223	4.669	7.482	5.710	5.807	4.977
Sign (difference from 0)	—	+++	++	—	+++	—

Contrary to the morphological involution of the lymph nodes, occurring after injections with cortisone acetate, there was a statistically highly significant increase in the accumulation of labelled phosphate per mg of tissue one hour after the administration of the phosphate (Table 4).

No significant change in the accumulation of P^{32} was found in the thymus or in the adrenals. In the spleen a highly significant

Table 4.

Change in accumulation of P^{32} (counts per minute and mg. of tissue) in guinea pigs injected with cortisone acetate in per cent of the accumulation in corresponding control animals. Paired comparisons, 19 pairs.

	Cervical lymph nodes	Inguinal lymph nodes	Spleen	Thymus	Adrenals
Mean increase of cortisone injected animals in per cent ..	+ 23.68	+ 25.58	+ 21.37	+ 10.84	+ 12.05
Standard error	8.180	6.520	5.279	8.036	17.17
Sign (difference from 0)	++	+++	+++	—	—

increase of P^{32} uptake was demonstrable. If the calculations were based on relative activities (relative to fat or muscle tissue) the increased accumulation of P^{32} in lymph nodes and in spleen proved to be still greater. Such calculations did not show any significant change in P^{32} uptake of the adrenals. In the thymus an almost significant increase of P^{32} accumulation was demonstrable in calculations based on relative activity.

No sexual differences were found as regards P^{32} accumulation in cortisone injected animals or in controls.

The differences in recorded radioactivity are not due to differences in self absorption, the latter having no influence bearing on counted impulses when the weight differences are not greater than the present (GYLLENSTEN and RINGERTZ 1954). This is also indicated by the fact that no significant increase of recorded activity per mg of tissue could be found as regards the thymus (Table 4), though the latter revealed a greater weight decrease than the lymph nodes (Table 3).

Discussion.

As argued by D'ANGELO et al. (1953) there may be differences in the reaction to exogenous cortisone between hypo-, eu- and hyperthyroid animals. The present investigations may confirm such a suggestion. Guinea pigs have naturally a low concentration of thyrotropic hormone in the blood and pituitary and a low thyroid activity (ADAMS 1946). In the present investigation they were found to react with thyroid stimulation on the administration of cortisone acetate. This contrasts with most reports on the

opposite reaction of the rat or human thyroid after cortisone administration. Rats, and to a less degree also human beings, are, however, hyperthyroid in comparison with guinea pigs. Our findings that guinea pigs given exogenous thyrotropic hormone show a depressed thyroid activation, judged from mean acinar cell height and P^{32} accumulation after the administration of cortisone acetate, seem consistent with the findings in rats and human beings.

The different results as to thyroid stimulation when judged from thyroid weight on one hand and from mean acinar cell height or P^{32} accumulation on the other, may be due to various components of the pituitary thyrotropic factor, one component influencing the weight, the other the acinar cells. Such differences have been discussed by, for instance, RAWSON and MONEY (1949), SALTER (1950), GYLLENSTEN (1953).

The correlation between P^{32} uptake and epithelial reaction (mean acinar cell height) confirms the statement of LAMBERG (1953) as to the equivalence of these two criteria.

According to RAWSON the thymus and lymphatic tissue have the capacity to inactivate thyrotropic hormone. The cortisone treatment caused a marked involution of the thymus and lymphatic tissue and would thus be expected to increase the thyroid sensitivity to exogenous thyrotropic hormone. However, the contrary was found, *i. e.*, a decreased thyroid reaction to thyrotropic hormone. As seen from the increase of P^{32} accumulation per mg of tissue in thymus and lymphatic tissue after cortisone administration, the quantitative involution might be compensated by increased metabolism, perhaps also then, by increased action on thyrotropic hormone per mg of tissue. Thus, the theory of RAWSON is not invalidated by the present findings.

Summary.

1. Intraperitoneal injections with 10 mg cortisone acetate per day for ten days in young guinea pigs produced thyroid activation, as judged from thyroid weight, mean acinar cell height and relative P^{32} accumulation.

2. Cortisone acetate, as previously mentioned, caused decreased thyroid stimulation by exogenous thyrotropic hormone when judged from mean acinar cell height and relative P^{32} accumulation but not when judged from thyroid weight.

3. The treatment with cortisone acetate caused an involution of thymus and lymph node tissue (by some 37 and 23 %, respectively) and an increased P^{32} accumulation per mg of tissue in the thymus, lymph node tissue and spleen (by some 11, 24, and 21 % respectively).

4. The findings are discussed with regard to the theory of RAWSON as to the inactivation of thyrotropic hormone in the thymus and lymphatic tissue.

References.

ABELIN, I., and U. ALTHAUS, Helv. chim. acta 1942. *25*. 205.

ADAMS, A. E., Quart. Rev. Biol. 1946. *21*. 1.

ALBERT, A., A. TENNEY, and E. FORD, Endocrinology 1952. *50*. 324.

ALTMAN, K. I., L. L. MILLER, and CH. G. BLY, Arch. Biochem. 1951. *31*. 329.

D'ANGELO, S. A., C. E. STEVENS, K. E. PASCHKIS, and A. CANTAROW, Proc. Soc. Exp. Biol. N. Y. 1953. *83*. 1. 181.

BEIERWALTES, W. H., W. Q. WOLFSON, J. R. JONES, C. T. KNORPP, and J. S. SIEMIENSKI, J. Lab. Clin. Med. 1950. *36*. 799.

BERSON, S. A., and R. S. YALOW, J. Clin. Endocr. Metabolism 1952. *12*. 407.

BESFORD, H., A. C. CROOKE, and J. D. MATTHEWS, Proc. Soc. Endocr., 29th ordin. Meeting, p. XV, J. Endocr. 1952. *8*.

BONNIER, G., and O. TEDIN, Biologisk variationsanalys, Bonniers förlag AB, Stockholm 1940.

BORELL, U., Acta med. Scand. 1945. Suppl. 161.

— and H. HOLMGREN, Acta endocr. Copenhagen 1949. *3*. 331.

BRIMBLECOMBE, R., C. P. HAIGH, I. D. K. HALKERSTON, M. REISS, and J. WARLEDGE, Proc. Soc. Endocr., 26th ordin. Meeting, p. V—VIII, J. Endocr. 1952. *8*.

EPSTEIN, D., A. CANTAROW, G. FRIEDLER, and K. G. PASCHKIS, Proc. Soc. Exp. Biol. N. Y. 1953. *82*. 50.

FRAENKEL-CONRAT, J., and C. H. LI, Endocrinology 1949. *44*. 487.

FREDERICKSON, D. S., P. H. FORSHAM, and G. W. THORN, J. Clin. Endocr. Metabolism 1952. *12*. 541.

GESCHWIND, I. I., C. H. LI, and H. M. EVANS, Endocrinology 1950. *47*. 162.

GYLLENSTEN, L., Acta anat. 1953. Suppl. 18 = 1 ad Vol. 17.

GYLLENSTEN, L., and N. R. RINGERTZ. Acta patholog. et microbiolog. Scandinav. In press. 1954.

HALMI, N. S., Proc. Soc. Exp. Biol. N. Y. 1952. *80*. 175.

— and S. B. BARKER, Endocrinology 1952. *51*. 127.

HIGGINS, G. M., K. A. WOODS, and E. C. KENDALL, Ibidem 1951. *48*. 175.

HILL, S. R., R. S. REISS, P. H. FORSHAM, and G. W. THORN, J. Clin. Endocr. 1950. *10*. 1375.

HOEN, A., H. LANGEFELD, and C. OEHME, Endokrinologie 1939. *21.* 305.
INGBAR, S. H., Endocrinology 1953. *53.* 2. 171.
KOELSCHE, G. A., and E. C. KENDALL, Amer. J. Physiol. 1935. *113.* 335.
LEDERER, J., Ann. Endocr. Paris 1952. *13.* 58.
LAMBERG, B.-A., Acta med. Scand. 1953. Suppl. 279.
MERCIER-PAROT, L., and H. TUCHMANN-DUPLESSIS, C. R. Soc. Biol. Paris 1951. *145.* 837.
MIGEON, C. J., L. I. GARDNER, J. F. CRIGLER, and L. WILKINS, Endocrinology 1952. *51.* 117.
MONEY, W. L., L. KIRSCHNER, L. KRAINTZ, P. MERRILL, and R. W. RAWSON, J. Clin. Endocr. 1950. *10.* 1282.
— L. KRAINTZ, J. FAGER, L. KIRSCHNER, and R. W. RAWSON, Endocrinology 1951. *48.* 6. 682.
OEHME, C., Klin. Wschr. 1936. *15.* 512.
PERRY, W. F., Endocrinology 1951. *49.* 284.
RAWSON, R. W., Ann. N. Y. Acad. Sci. 1949. *50.* 491.
— Ciba Foundation Colloquia on Endocrinology 1952. *IV.* 294.
— G. D. STERNE, and J. C. AUB, Endocrinology 1942. *30.* 240.
— R. M. GRAHAM, and C. B. RIDDELL, Ann. Int. Med. 1943. *19.* 405.
— and W. L. MONEY, Rec. Progr. Hormone Res. Vol. IV. 397. 1949. Acad. Press New York.
SALTER, W. T., Chapter IV and V in: PINCUS, G., and K. V. THIMANN: The hormones, Vol. 2, New York 1950, Acad. Press Inc.
SELYE, H., Stress, Acta, Inc., Medical Publishers, Montreal, Canada 1950.
— First annual report on stress, Ibid. 1951.
— and A. HORAVA, Second annual report on stress, Ibid. 1952.
— — Third annual report on stress, Ibid. 1953.
SHELLABERGER, S., Anat. Rec. 1953. *117.* 552.
SPIRTOS, B. N., Proc. Soc. Exp. Biol. N. Y. 1953. *84.* 3. 673.
VERZAR, F., and V. VIDOVIC, Helv. physiol. acta 1951. *9.* 214.
WINTER, C. A., R. H. SILBER, and H. C. STOERK, Endocrinology 1950. *47.* 60.
WOLFSON, W. Q., W. H. BEIERWALTES, W. D. ROBINSON, I. F. DUFF, J. R. JONES, C. T. KNORPP, and MIOKO EYA, J. Lab. Clin. Med. 1950. *36.* 1005.
WOODBURY, D. M., B. N. GHOSH, and G. SAYERS, J. Clin. Endocr. 1951. *11.* 761.

From the Institute of Organic Chemistry and Biochemistry, University of Stockholm, Sweden.

The Occurrence of Choline Esters in the Honey-Bee.

By

KLAS-BERTIL AUGUSTINSSON and MARGARETA GRAHN.

Received 26 April 1954.

The occurrence in insects of choline esters other than acetylcholine can be inferred from studies of insect enzymes. The presence of an active principle similar to acetylcholine has been demonstrated by various authors (CORTEGGIANI and SERFATI 1939, MIKALONIS and BROWN 1941, TOBIAS, KOLLROS and SAVIT 1946, FERNANDO 1951, YUSHIMA and CHINO 1953). The identification of this substance as acetylcholine was more conclusively established by LEWIS (1953).

The insect nervous system has a comparatively high cholinesterase activity, upon which the nervous function of these animals is dependent (MEANS 1942, ROEDER, KENNEDY and SAMSON 1947, ROCKSTEIN 1950). Insect cholinesterase differs in some respects from the cholinesterase of higher animals. Thus, it was demonstrated that acetyl-β-methylcholine is split by the cholinesterase of certain insects more rapidly than acetylcholine (RICHARDS and CUTKOMP 1945, TOBIAS *et al.* 1946, cf. AUGUSTINSSON 1948). A more detailed study of the substrate specificity was carried out by METCALF and MARCH (1950) and by BABERS and PRATT (1951). These authors found distinct differences in the cholinesterases from the brain of the house-fly, the honey-bee, and the mouse. These differences are reflected in the kinetics of their cholinesterase activity towards acetyl-β-methylcholine. The hypothesis (AUGUSTINSSON 1948) put forward to explain the fact that this substrate

is split at a higher rate than acetylcholine by the brain of honey-bee has been confirmed in their experiments. They found that at high substrate concentrations ($> 10^{-2}$ *M*) the hydrolysis of acetyl-β-methylcholine is greater than that of acetylcholine, at low substrate concentrations ($< 10^{-2}$ *M*) the reverse is true. In the case of the house-fly, acetylcholine is hydrolysed faster than acetyl-β-methylcholine at all concentrations. Whether one enzyme (METCALF and MARCH 1950) or two enzymes (BABERS and PRATT 1951) are responsible for the hydrolysis of the two substrates by the honey-bee brain has not yet been established conclusively. The high rate of metabolism of acetyl-β-methylcholine by honey-bee brain could be regarded as an indication that this ester is a natural substrate for cholinesterase in the honey-bee.

Since the time of the classical work of LOEWI, DALE, GADDUM, FELDBERG, NACHMANSOHN and others, little attention has been directed toward the problem of whether choline esters other than acetylcholine are present in animal tissues and play a rôle in nervous function. Biological methods for the assay of acetylcholine are not specific, insofar as the compound which is actually assayed is concerned. The introduction of new micro methods has made possible rapid advances in the separation and identification of biologically active compounds present in tissues in very small amounts. One of these methods, paper chromatography, has been shown to be suitable for the separation of various esters of choline (WHITTAKER and WIJESUNDERA 1952, AUGUSTINSSON and GRAHN 1953). Using this method in combination with classical bioassay methods, BANISTER, WHITTAKER and WIJESUNDERA (1953) recently demonstrated that ox spleen contains acetylcholine, propionylcholine and a third unidentified choline ester. These results have recently been confirmed in our laboratory. Another successful identification of an ester of choline other than acetylcholine was recently announced by ERSPAMER and BENATI (1953). The highly active murexine, which is present in the hypobranchial gland of *Murex trunculus* and has been known since 1938 to be closely related to acetylcholine, was identified as urocanylcholine (or β-/imidazolyl-4/-acrylcholine).

In the present study the occurrence of choline esters in the head of the honey-bee was investigated. Active fractions of extracts from this material were analysed by paper chromatography using both chemical and biological methods for the identification of choline esters. In this way it was demonstrated that in addition

to acetylcholine the head of the honey-bee contains at least one other choline ester; this ester has not yet been identified, but it is not acetyl-β-methylcholine.

Methods and Material.

Chemical estimation of choline esters was carried out by the hydroxylamine-ferric chloride test described by HESTRIN (1949).

Paper chromatography was carried out according to the method recently described (AUGUSTINSSON and GRAHN 1953). The solvent used was a *n*-butanol-ethanol-acetic acid-water mixture (8:2:1:3). The ascending technique was used except as otherwise stated.

Pharmacological assays. The guinea-pig ileum was used as a test object in the usual way by suspending a 2—3 cm strip in an organ bath (20 ml) of Tyrode solution, the composition of which was that described by BENTLEY and SHAW (1952). That solution contained a higher concentration of calcium chloride (2.0 g/l) than earlier descriptions. The bath was oxygenated and maintained at 30° C.

Preparation of extracts. Thousands of honey-bees were killed by spraying with a solution of parathion to be sure that all cholinesterase activity was destroyed. In two preliminary small-scale experiments, 5,000 and 6,000 bees were used. The main experiment, which will be described in detail, was carried out using 22,000—23,000 bees. The animals were decapitated and the heads (258 g) were treated with 850 ml of 10 % trichloroacetic acid and homogenized at high speed (BÜHLER 7073). On the following day the extract was filtered and the residue washed with 7 % trichloroacetic acid (altogether 550 ml). The filtrate was repeatedly extracted with ether (final pH 3.5) and evaporated. It was found necessary to extract with ether once or twice during the evaporation procedure to remove the trichloroacetic acid. The final solution (at pH 3.5—4) was treated with an equal volume of ethanol (acidified with hydrochloric acid to pH 4), filtered, the residue washed, and the filtrate evaporated. Evaporation was always carried out at a temperature not exceeding 40° C. The extract was subjected to a chemical test (HESTRIN's method) and paper chromatography, and was used for the isolation of the tissue bases with Reinecke salt and sodium tetraphenylboron.

Isolation of tissue bases as reineckates. The procedure of KAPFHAMMER and BISCHOFF (1930) was followed with slight modifications. The water extracts were treated with Reinecke salt dissolved in water acidified with hydrochloric acid to pH 4 (1.5 g Reinecke salt per 100 ml). After standing in the refrigerator for an hour the precipitate was filtered off and the filtrate reprecipitated with Reinecke salt. The precipitation procedure was carried out using 20 ml aliquots of the extracts. After washing with ice water, ethanol and ether the precipitate (1.5 g) was dissolved in aqueous acetone (pH 4) and the solution treated with silver sulphate (saturated solution) to slight excess of silver. The silver reineckate was removed by filtration and the colourless filtrate precip-

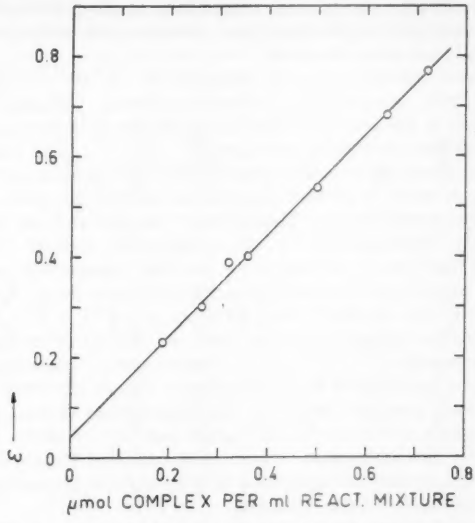


Fig. 1. Calibration curve for the colorimetric determination of acetylcholine in the form of its tetraphenylboron complex. The method is based on the hydroxylamine-ferric chloride test.

itated with barium chloride (1 % solution) for complete removal of SO_4^{2-} and Ag^+. The slight excess of Ba^{2+} was removed with sodium sulphate, the precipitate filtered off, and the solution evaporated to a small volume. The solution was tested biologically (on guinea-pig ileum), chemically (with hydroxylamine-ferric chloride), and was chromatographed on paper by the method described above.

Isolation of tissue bases as tetraphenylboron complexes. MARQUARDT and VOGG (1952) have reported that acetylcholine and choline can be determined gravimetrically by precipitation with sodium tetraphenylboron ("Kalignost"). This reagent has been used in the quantitative determination of potassium (WITTIG, KEICHER, RÜCKERT and RAFF 1949, RAFF and BROTZ 1951). It was tested in this laboratory for the isolation of choline and choline esters from tissue extracts and extracts of certain areas of paper chromatograms. Preliminary experiments were carried out with pure compounds. Sodium tetraphenylboron (mol. wt. 342.2) was used as a 0.1 *M* aqueous solution. The aqueous solutions of choline and choline esters were made acid to pH 4 with acetic acid and the reagent added dropwise to excess. After 24 hours in the refrigerator the mixture was centrifuged and the precipitate washed three times with water containing a few drops of acetic acid. When centrifugation did not give a complete separation of the precipitate a small amount of kieselgur was added. The complex salt was dissolved in pure acetonitrile; acetone can also be used, but it is not as suitable as the former solvent

(cf. below). Choline esters were determined as their tetraphenylboron complex using hydroxylamine-ferric chloride test and acetylcholine tetraphenylboron as a standard.

Colorimetric determination of acetylcholine in the tetraphenylboron complex. Pure acetylcholine tetraphenylboron, $C_7H_{16}O_2/B(C_6H_5)_4/$, was prepared in the way described; acetylcholine is isolated in this procedure in almost theoretical amounts (95—100 %). The complex was recrystallized from acetone and carefully dried; the pure compound melts at 180° C. A solution of this complex in acetonitrile gives a positive colour reaction with hydroxylamine-ferric chloride and can be used for acetylcholine determination by this colorimetric method. Acetonitrile must be of high purity; acetone is not suitable because it gives a precipitate with alkaline hydroxylamine and hydrochloric acid. A calibration curve (Fig. 1) was obtained using dilutions of a 4.46×10^{-3} *M* solution of acetylcholine tetraphenylboron (mol. wt. 465.4) in acetonitrile.

Chromatographic characterization of choline esters. Concentrated tissue extracts, and solutions of tetraphenylboron complexes, reineckates and chlorides were chromatographed. The appropriate areas, determined by developing a reference chromatogram (see below), were cut out and used directly for bioassay or eluted with acidified water. The eluates were used for re-chromatographing or for chemical estimation of esters with hydroxylamine-ferric chloride.

The paper chromatograms were developed by spraying with dipicrylamine (AUGUSTINSSON and GRAHN 1953), hydroxylamine-ferric chloride (WHITTAKER and WIJESUNDERA 1952), or *p*-diazobenzolsulphonic acid (PAULY's reagent, for the detection of histamine).

Biological characterization of choline esters on paper chromatograms. In preliminary experiments the various areas were eluted with Tyrode solution or, better, with acidified water, and the extracts tested biologically on isolated guinea-pig ileum (see above). It is more convenient to put the paper strip containing a certain area of the chromatogram directly into the organ bath. The reaction of the ileum is slower, but it is a much more rapid way to test the activity on the chromatogram.

Special chemicals. Choline and its esters were used as chlorides, unless otherwise stated. Sodium tetraphenylboron, $Na/B(C_6H_5)_4/$, was a commercial product (trade name "Kalignost", manufactured by Messrs. HEYL & Co., Hildesheim, Germany). As solvent for the complexes formed with this agent acetonitrile of high purity was used; it was shaken with saturated potassium carbonate and redistilled several times with P_2O_5 (b.p. 82° C, 1 atm.). The Reinecke salt, $NH_1/Cr\ (NH_3)_2\ (SCN)_1/.\ H_2O$, was a commercial product (Eastman Kodak). Other chemicals used were of high purity grade.

Results.

Chromatography of extracts of tissue bases. Concentrates of tissue bases, prepared according to the method described above, were paper chromatographed and the chromatograms developed with

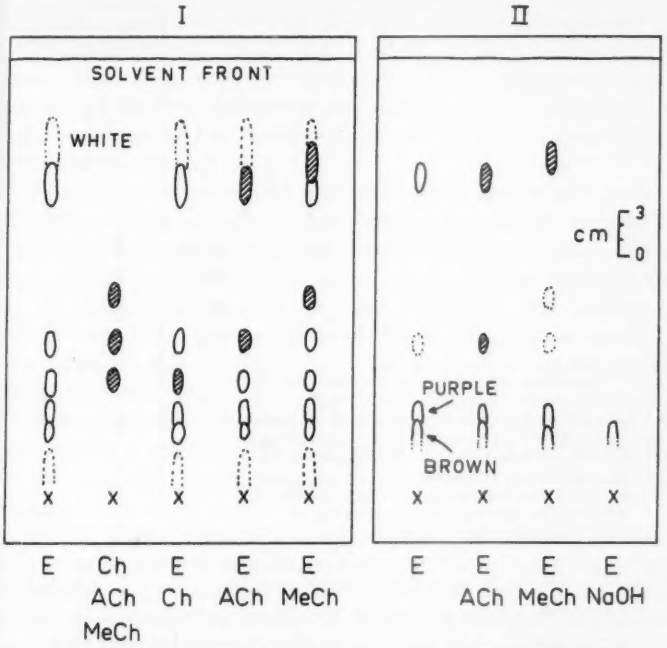


Fig. 2. Ascending chromatograms of an extract (E) of concentrated tissue bases. The chromatograms are developed with dipicrylamine (I) and hydroxylamine-ferric chloride (II). Solvent, *n*-butanol-ethanol-acetic acid-water (8:2:1:3); run, 30 cm; R_F of choline chloride (Ch), acetylcholine chloride (ACh), and acetyl-β-methylcholine chloride (MeCh), 0.27, 0.35, and 0.46.

dipicrylamine and hydroxylamine-ferric chloride. On such chromatograms a number of spots are visible. These were analysed by chromatographing extracts mixed with pure compounds (Fig. 2). It was found that acetylcholine and acetyl-β-methylcholine, added to the extract, are not confined to the areas expected, but also occur in areas with higher R_F. Similar results were obtained with extracts of other tissues (ox spleen, dog brain) prepared by the same technique (using trichloroacetic acid). All chromatograms developed with dipicrylamine are characterized by a white area of R_F 0.80. This is due to a decolourization by trichloroacetic acid, which is always present in small quantities, even after repeated treatment of the extracts with ether. Esters of choline added to the extracts are found in the vicinity and partly overlapping this white area.

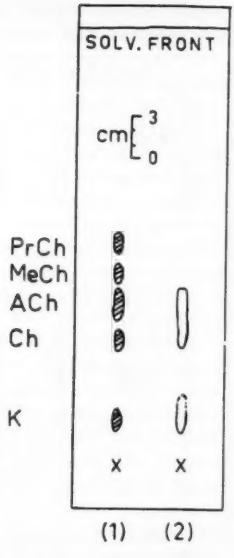


Fig. 3. Ascending chromatogram of a solution (in acetonitrile) of pure tetraphenylboron complexes (1) as compared with a mixed complex (2) obtained from an extract of concentrated tissue bases treated with sodium tetraphenylboron. R_F: potassium 0.10, Ch 0.29, ACh 0.37, MeCh 0.44, PrCh (propionylcholine) 0.51. The symbols in this figure (cf. Fig. 2) refer to the corresponding complexes of tetraphenylboron.

From the dipicrylamine chromatograms (Fig. 2, I) it is concluded that acetylcholine is present in the tissue extracts and is visible as two areas (R_F 0.35 and 0.72) and that acetyl-β-methylcholine is not present. Choline is found in the area expected from its known R_F (0.27). The colour of this area is generally pinker than the colours given by the choline esters. Chromatograms developed with hydroxylamine-ferric chloride (Fig. 2, II) confirm these results. The latter chromatograms are not as clear as the dipicrylamine chromatograms.

In addition to the areas mentioned above there are two areas with lower R_F, which are visible on both the dipicrylamine and hydroxylamine-ferric chloride chromatograms (Fig. 2). In the latter case the area of lower R_F (0.15) is brown and that of the higher R_F (0.19) blue-purple. It was demonstrated that the brown area corresponds to histamine (see below).

Chromatography of tissue bases isolated as tetraphenylboron complexes. A mixture of the chlorides of choline, acetylcholine, acetyl-β-methylcholine, propionylcholine, and potassium was precipitated with sodium tetraphenylboron and the precipitate dissolved in pure acetonitrile. This solution was chromatographed and developed with dipicrylamine. It was found (Fig. 3) that all

of these substances resolved from one another. An extract of concentrated tissue bases was treated similarly with sodium tetraphenylboron and the precipitate chromatographed. Comparison of the two chromatograms showed that the unknown solution contains choline, acetylcholine, potassium, and probably an unknown compound (R_F 0.21).

Pure choline esters can be precipitated completely with sodium tetraphenylboron at pH 4. The precipitates formed are rather difficult to centrifuge (especially that of propionylcholine). They must be carefully washed, for small amounts of the precipitating agent disturb the quantitative determination with hydroxylamine-ferric chloride. A solution of the complexes in acetonitrile is suitable for this colorimetric method, and the esters are almost completely recovered.

When a solution of pure acetylcholine tetraphenylboron in acetonitrile is applied on a filter paper, air-dried and eluted, 85 to 90 % of the original amount of the complex is found in the eluate.

A solution of a mixture of the chlorides of choline, acetylcholine, and propionylcholine was chromatographed in the usual way. One paper strip used as control was developed with dipicrylamine. The three compounds were traced at R_F 0.29 (choline), 0.39 (acetylcholine), and 0.48 (propionylcholine). The corresponding areas of another (non-developed) strip were eluted and the eluates treated with sodium tetraphenylboron. There was good precipitation in all three solutions. The precipitates were dissolved in acetonitrile and the solutions analysed quantitatively for esters. The recovery of acetylcholine was 33—45 % and that of propionylcholine 25—40 %.

Based on these preliminary results the following experiment was carried out with the extract of concentrated tissue bases from the honey-bee material. The extract was chromatographed after application of a 26 cm long band (22 mm wide) of drops to the paper. On each side of this band the extract was applied as a drop in the usual way to be used as reference chromatograms developed with dipicrylamine. After running for 16 hours the part of the chromatogram intended for extraction was divided into nine sections. Each section was carefully eluted with distilled water (acidified to pH 4) and the eluate precipitated with sodium tetraphenylboron (0.1 *M* aqueous solution). The solutions of the washed precipitates in acetonitrile were analysed with hydroxyl-

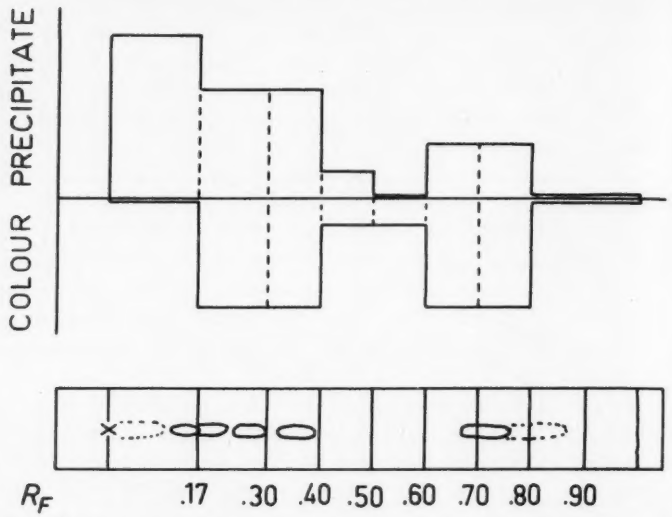


Fig. 4. Tracing of the components of concentrated tissue bases with sodium tetraphenylboron. Each section, noted on the reference chromatogram, was eluted and precipitated with sodium tetraphenylboron. The precipitates were dissolved in acetonitrile and analysed colorimetrically with hydroxylamine-ferric chloride. The relative amounts of precipitates formed and colour intensities are given for each section of the chromatogram. The reference chromatogram was developed with dipicrylamine.

amine-ferric chloride. Fig. 4 shows the relative amount of precipitate formed from each section of the chromatogram and the relative colour intensities given by the solutions of the precipitates. The heavy precipitate from the first section (R_F 0—0.17) was due to monovalent cations, mainly potassium; it therefore gave no colour reaction. The precipitates from the other sections gave positive colour reactions indicating the presence of esters. The positive colour reaction of the section (R_F 0.17—0.30) which is believed to contain both choline and the unknown ester (mentioned above), confirms the suggestion that there is a choline ester present in this area (R_F 0.19), although it is difficult to measure the exact amount.

Chromatography of tissue bases isolated as reineckates and chlorides. The tissue bases were precipitated from the water extracts with Reinecke salt. The reineckate precipitates were dissolved in aqueous acetone (pH 4) and used in preliminary chromatography experi-

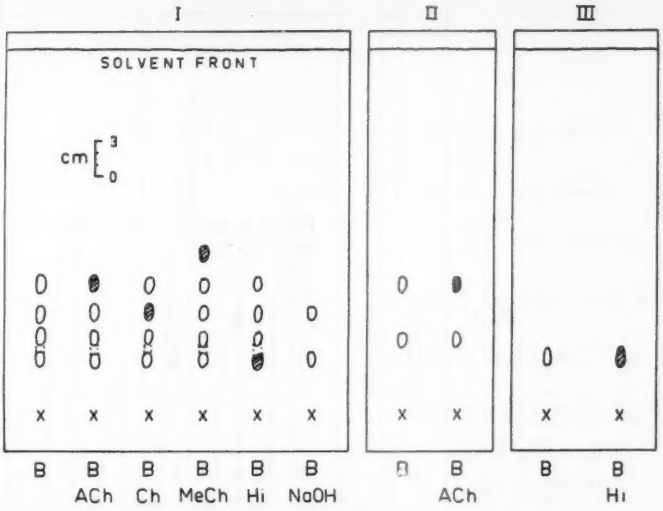


Fig. 5. Ascending chromatograms of tissue bases isolated as chlorides (B). The chromatograms are developed with dipicrylamine (I), hydroxylamine-ferric chloride (II), and *p*-diazobenzolsulphonic acid (for histamine) (III). R_F: ACh 0.36, Ch 0.28, MeCh 0.45, histamine dihydrochloride (Hi) 0.15. Symbols as in Fig. 2.

ments. Chromatography splits the reineckates (except that of choline) into the free base and Reinecke reagent; BREGOFF, ROBERTS and DELWICHE (1953) recently observed a similar splitting of the reineckates of a series of quaternary ammonium bases. On a chromatogram developed with dipicrylamine, a mixture of the reineckates obtained from pure choline esters gave satisfactory separation of the individual compounds; the corresponding R_F values were close to those of the chlorides. There were, however, difficulties in getting clear chromatograms with the reineckates precipitated from tissue extracts. We therefore converted the reineckates to the chlorides by the usual method with silver sulphate and barium chloride.

The aqueous solution of the chlorides prepared from the tissue bases was chromatographed and developed with dipicrylamine, hydroxylamine-ferric chloride and the histamine reagent (according to PAULY). On the dipicrylamine chromatogram four areas are visible (Fig. 5, I) which were analysed by comparison with pure compounds. The area of the highest R_F (0,37) is acetylcholine,

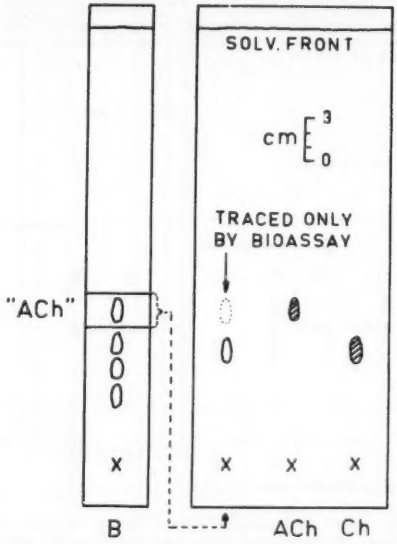


Fig. 6. Re-chromatography of the acetylcholine area ("ACh"). Ascending chromatograms of a solution of isolated tissue bases (B) (as chlorides). During the elution of the original acetylcholine area with Tyrode solution most of the ester suffered hydrolytic decomposition. Therefore, after re-chromatography acetylcholine was barely detected with dipicrylamine, but could still be readily detected by bioassay.

followed by choline (R_F 0.28). The slowest running compound (R_F 0.16) was identified as histamine. The fourth area (R_F 0.20) could not be identified, but it disappeared after treatment with alkali. This compound is believed to be a new choline ester, for it is found on a chromatogram developed with hydroxylamine-ferric chloride (Fig. 5, II). The four compounds found on the dipicrylamine chromatogram were also found on chromatograms obtained with the crude extract described above (Fig. 2, I). The histamine was confirmed with PAULY's technique.

In a preliminary experiment the acetylcholine area ("ACh", Fig. 6) was eluted with Tyrode solution and the eluate re-chromatographed. After developing with dipicrylamine hardly any material was found in the acetylcholine position; instead there was a deeply coloured area corresponding to choline. However, acetylcholine was detected at its expected position using bioassay with isolated guinea-pig ileum. The activity of the eluate of the "new"

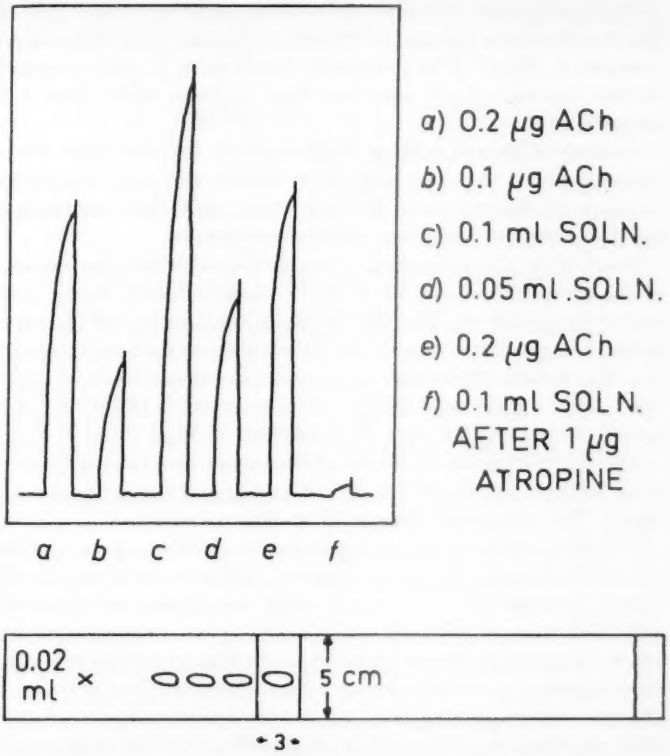


Fig. 7. Bioassay (guinea-pig ileum) carried out on the acetylcholine area of an ascending chromatogram. The original test solution was that of the isolated tissue bases (as chlorides). Contractions of the isolated ileum were compared with those produced by known amounts of acetylcholine chloride (ACh). Total volume of the eluate (in acidified water), 5.0 ml, corresponds to 0.02 ml of the original test solution.

acetylcholine area was completely blocked after the addition of atropine sulphate (0.5 μg) to the organ bath. The activity also disappeared completely after incubation of the eluate with purified preparations of acetylcholinesterase (electric tissue) and human plasma cholinesterase. The biological activity could also be destroyed by short boiling with alkali and subsequent neutralization. Because elution of acetylcholine with Tyrode solution leads to an appreciable hydrolysis of the ester this method was not used in subsequent bioassay or re-chromatography studies.

Chromatograms were also eluted with acidified water (pH 4). The results of the bioassay of eluates of the acetylcholine area are recorded in Fig. 7. The compound eluted gave a positive acetylcholine reaction in all tests and there is little doubt that it is acetylcholine.

Instead of always making eluates of the various areas of the chromatograms the areas were often cut out and used directly for bioassay by dipping them into the organ bath. This technique is rapid, convenient and gives satisfactory results.

Descending chromatography was employed when the bioassay of the compounds found between the histamine and choline areas was to be carried out. The solvent was allowed to run off the paper in order to get better separation. In addition to the histamine area (see Fig. 8) two other areas were visible on chromatograms developed with dipicrylamine. One of these areas (3) (R_F 0.20) corresponds to the choline ester demonstrated in Figs. 2 and 5. Strips of the chromatograms containing these areas were cut into one-cm wide sections and the biological activity tested against guinea-pig ileum. The results are illustrated in Fig. 8.

All three compounds running slower than choline give contraction of the isolated ileum. As expected, the activity of the slowest running compound (1) (R_F 0.15), which was shown to be identical with histamine (cf. Fig. 5), was blocked by an antihistamine but not by atropine. It is seen by reference to Fig. 8 that the two other areas appear to contain choline esters. The biological activity of the fast running compound (3) was destroyed after incubation with solutions of cholinesterases (top of Fig. 8). This compound is believed to be a new choline ester. It is hydrolysed by both acetylcholinesterase (electric tissue) and plasma cholinesterase. The other compound (2), also believed to be a choline ester, was not studied enzymatically.

The amount of acetylcholine and other choline esters present in the head of the honey-bee. Quantitative calculations are difficult because the fractionation and isolation procedures were stepwise. Furthermore, the fractions were consumed by preliminary experiments, which were necessary to ascertain the best experimental conditions. There is a great loss of material during the experimental procedures, especially through hydrolytic breakdown of active choline esters. In each procedure, particularly during the elution of chromatograms, there is an appreciable increase in free choline. Every possible effort to minimize this loss has been made, but we

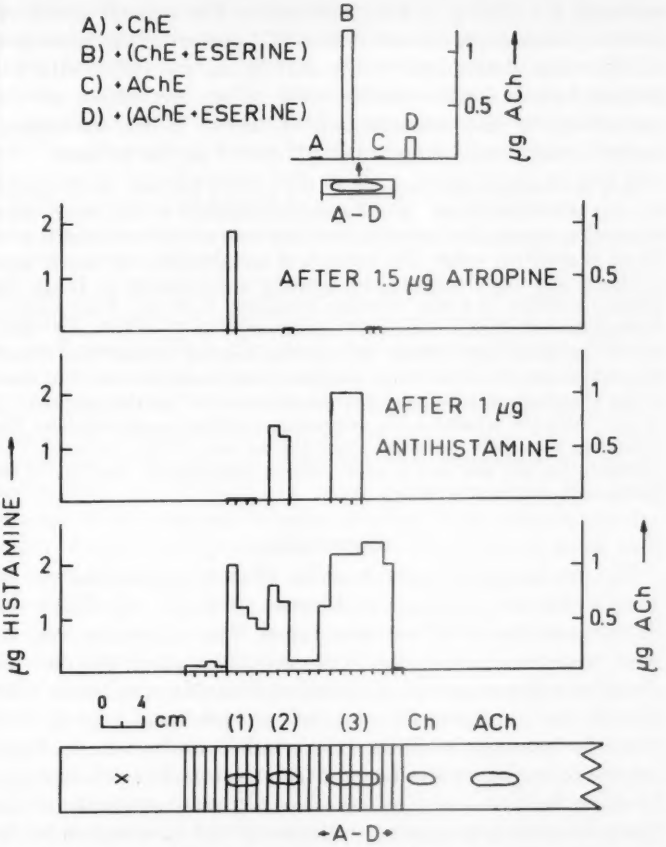


Fig. 8. Bioassay of the various components visible on a chromatogram developed with dipicrylamine. The components were separated by descending chromatography. One-cm-wide sections of the chromatograms were tested directly by dipping them into the organ bath. The effects of antihistamine ("lergigan") and atropine sulphate on the biological activity were tested. A—D refers to four identical areas of the unknown choline ester (3), tested after incubation with purified human plasma cholinesterase (ChE) and purified electric tissue acetylcholinesterase (AChE); these preparations were used before and after enzyme inhibition performed by the addition of eserine sulphate. (1) histamine; (2) and (3) refer to the other two components studied.

do not believe that more than about 10—20 % of the original activity is found in the final solutions.

Altogether 1.5 g of reineckate was obtained from the original

material, *i. e.*, 258 g of honey-bee heads. The amount of acetylcholine chloride equivalents ("ACh-Cl") present in the reineckate mixture was determined to be 0.16 % as measured with the hydroxyl-amine-ferric chloride test (after conversion of the reineckates to the corresponding chlorides). Hence, the original material contained 2.4 mg "ACh-Cl" or 9.3 μg per g tissue.

In a chromatographic experiment 0.2 ml of a solution (total volume 4.4 ml) of isolated bases (as chlorides) was applied to the paper. After chromatographing, the acetylcholine area was cut out and eluted with 10 ml of acidified water. The amount of acetylcholine chloride present in this eluate was determined by bioassay and found to be 15 μg; the original solution (4.4 ml) therefore contained 0.33 mg. That solution corresponds to 1.22 g reineckate, containing 0.16 % or 1.95 mg "ACh-Cl". From these approximate calculations, keeping in mind that elution of acetylcholine from the chromatogram is not complete and that there is loss of activity due to hydrolytic breakdown, we find that about 17 % of the "ACh-Cl" present in the reineckate mixture is acetylcholine. The remaining esters are probably other choline esters. The value 17 % is probably far too low and a more reliable quantitative analysis of the choline esters is to be carried out.

Discussion.

The precise physiological function of acetylcholine has not yet been established, although it is known to play a significant rôle in the operation of the nervous system. This is probably true for most types of nervous tissue in the animal kingdom. The presence of acetylcholinesterase in all nervous tissues (NACHMANSOHN 1952) favours this hypothesis, but cannot be regarded as a proof of it. Thus, for example, esterases exist which hydrolyse other choline esters at a higher or similar rate than they do acetylcholine, and in many instances acetylcholine has not been positively identified as the natural substrate. Moreover, the distribution of the acetylcholine-cholinesterase system is not closely correlated with nervous function. The relatively high content of acetylcholine in non-conducting or nerve free organs (spleen, placenta) and the high cholinesterase activity of such tissues as erythrocytes, placenta, snake venom, and *Helix* blood can hardly be concerned with nervous propagation.

The rôle played by acetylcholine or other choline esters in the insect nervous system has not yet been conclusively demonstrated. Despite the high concentrations of acetylcholine and cholinesterase in the nervous system of many insects (for literature references, see the introduction), the nerve centres of these animals

are relatively insensitive to acetylcholine, both in the presence and absence of cholinesterase inhibitors (ROEDER and ROEDER 1939, ROEDER 1948, ROEDER, KENNEDY and SAMSON 1947). As was pointed out in the introduction, enzyme studies suggest the possibility that another choline ester, acetyl-β-methylcholine, may be a natural substrate for cholinesterase in certain insects. The present investigation was, in fact, undertaken to test the possible occurence of other esters of choline in honey-bees.

In the head of the honey-bee two biologically active components were found in addition to acetylcholine. They resemble acetylcholine both biologically and chemically and they are believed to be new esters of choline. One of the compounds was found both in crude concentrated extracts and in mixtures of isolated tissue bases. It was studied enzymatically and found to be destroyed by cholinesterases. Neither of the compounds has yet been identified, but acetyl-β-methylcholine has been ruled out. Acetylthiocholine is not present. Both the new compounds run more slowly than acetylcholine on paper chromatograms. The acid moieties of the esters are therefore believed to be of low molecular weight. Formic acid, glycine or a simple derivative of acetic acid are possibilities but aromatic acids are not present.

Acetylcholine was demonstrated with certainty in the honey-bee head and it is present in potentially effective concentrations. The rôle played by acetylcholine in these animals has never been convincingly demonstrated and nothing further can be concluded about it from these studies. Nor is it possible to define the rôle played by the other choline esters which are present. Further examination of the actions of chemical agents belonging to the choline ester group and of cholinesterase inhibitors in honey-bees and other species of insects are to be carried out. A detailed study of the new choline esters will also be made.

Summary.

The occurrence of choline esters in the head of the honey-bee was investigated. Paper chromatography was used for the separation of these compounds.

The tissue bases were isolated as the complex salts of tetraphenylboron and as reineckates. They were characterized and assayed by chemical and biological methods.

In addition to acetylcholine, the head of the honey-bee contains at least one other ester of choline and there is probably also a

third choline ester present. One of these esters was studied in more detail, but neither has been identified. Acetyl-β-methylcholine is not present in these insects.

This work was supported by a grant from *Statens Naturvetenskapliga Forskningsråd* (*Swedish Natural Science Research Council*). We wish to express our sincere thanks to Assistent BERTIL SCHWAN, Sveriges Husdjursförsök, Uppsala, for supplying the honey-bees.

References.

AUGUSTINSSON, K.-B., Acta physiol. Scand. 1948. *15*. Suppl. 52.
— and M. GRAHN, Acta chem. Scand. 1953. *7*. 906.
BABERS, F. H., and J. J. PRATT JR., Physiol. Zool. 1951. *24*. 127.
BANISTER, J., V. P. WHITTAKER, and S. WIJESUNDERA, J. Physiol. 1953. *121*. 55.
BENTLEY, G. A., and F. H. SHAW, J. Pharmacol. 1952. *106*. 193.
BREGOFF, H. M., E. ROBERTS, and C. C. DELWICHE, J. biol. Chem. 1953. *205*. 565.
CORTEGGIANI, E., and A. SERFATY, C. R. Soc. Biol. Paris 1939. *131*. 1124.
ERSPAMER, V., and O. BENATI, Biochem. Z. 1953. *324*. 66.
FERNANDO, H. E., Doctoral Thesis, University of Illinois, Urbana, Ill. 1951.
HESTRIN, S., J. biol. Chem. 1949. *180*. 249.
KAPFHAMMER, J., and C. BISCHOFF, Hoppe-Seyl. Z. physiol. Chem. 1930. *191*. 179.
LEWIS, S. E., Nature 1953. *172*. 1004.
MARQUARDT, P., and G. VOGG, Hoppe-Seyl. Z. physiol. Chem. 1952. *291*. 143.
MEANS, O. W. JR., J. cell. comp. Physiol. 1942. *20*. 319.
METCALF, R. L., and R. B. MARCH, J. econ. Entomol. 1950. *43*. 670.
MIKALONIS, S. J., and R. H. BROWN, J. cell. comp. Physiol. 1941. *18*. 401.
NACHMANSOHN, D., Bull. Soc. Chim. biol. 1952. *34*. 447; Rend. Ist. super. Sanità 1952. *15*. 1266.
RAFF, P., and W. BROTZ, Z. anal. Chem. 1951. *133*. 241.
RICHARDS, A. G. JR., and L. K. CUTKOMP, J. cell. comp. Physiol. 1945. *26*. 57.
ROCKSTEIN, M., Ibidem 1950. *35*. 11.
ROEDER, K. D., Bull. Johns Hopkins Hosp. 1948. *83*. 587.
— Insect Physiology. Wiley & Sons, New York 1953.
— N. K. KENNEDY, and E. A. SAMSON, J. Neurophysiol. 1947. *10*. 1.
— and S. ROEDER, J. cell. comp. Physiol. 1939. *14*. 1.
TOBIAS, J. M., J. J. KOLLROS, and J. SAVIT, Ibidem. 1946. *28*. 159.
WHITTAKER, V. P., and S. WIJESUNDERA, Biochem. J. 1952. *51*. 348.
WITTIG, G., R. KEICHER, A. RÜCKERT, and P. RAFF, Liebigs Ann. 1949. *563*. 110; cf. Angew. Chem. 1950. *62*. 231.
YUSHIMA, T., and H. CHINO, Oyo-Kontyu 1953. *9*. 52; abstracted in Chem. Abstr. 1954. *48*. 1592.

From the Department of Chemistry, the Royal Veterinary College, Stockholm.

Phyrophosphatase from Horse Erythrocytes. Inorganic Inhibitors and Activators.

By

KNUT SJÖBERG.

Received 26 April 1954.

In a previous paper the author has given an account of experiments with pyrophosphatase from erythrocytes in man and different kinds of animals. It was then shown that blood plasma contains a substance that inhibits very much the action of pyrophosphatase. This substance is thermostabile and dialyzes through a collodium membrane. In order to determine the nature of the inhibitory substance, the author has investigated the effect of a number of inorganic ions on the action of pyrophosphatase. Pyrophosphatases of different origin and specificity are inhibited or activated in different ways. One can distinguish between two main groups of pyrophosphatases from animal tissues. According to ROCHE and coworkers (1944), pyrophosphatase with an optimum pH of from 7.0—7.2 is activated by Mg^{2+} and other bivalent metal ions (Zn, Mn, Co, Fe). According to UZAWA (1936), BAUER (1936), and FLEURY and COURTOIS (1937), pyrophosphatases with an optimum pH of from 5.0—5.5 are less dependent on bivalent ions but are very much inhibited by F^-. According to NAGANNA and MENON (1948), pyrophosphatases from erythrocytes (homo) are activated by Mg^{2+} and small amounts of CN^- but are inactivated completely by 0.01 M Ca^{2+} and small amounts of heavy metals and fluorides. According to GORDON (1950), pyrophosphatase from brain, yeast and erythrocytes is inhibited by inhibitors which react with SH groups and by Ca^{2+}, F^- and CN^-. On the other hand,

it is activated by Mg^{2+}. HEPPEL and HILMOE (1951) have found that pyrophosphatase from bakers' yeast and bull sperm is activated by small amounts of Mg^{2+}. According to SWANSSON (1952), pyrophosphatase from rat liver homogenate is activated by Mg^{2+} up to 0.02 M but inactivated by Ca^{2+} and F^-, whereas ATPase, according to several investigators, is inactivated by Mg^{2+} and activated by Ca^{2+} (MOMMAETS and SERAIDARIAN 1947, ENGELHARDT and coworkers 1941, BAILEY 1942, SPICER and BOWEN 1951, CSAPÓ 1949, GERGELY 1953, and others). On the other hand, MEYERHOF and coworkers (1948, 1952) state that a myocin-free ATPase activated by Mg^{2+} and inactivated by Ca^{2+} has been isolated from muscles. Several investigators have also found an inhibition of ATPase with heavy metal ions (Ag^+, Cu^{2+}) (ENGELHARDT and coworkers 1941, BAILEY 1942, ZIFF 1944, SINGER and BARRON 1944). The pyrophosphatases from erythrocytes from different kinds of animals examined by the present author are activated by Mg^{2+} and have an optimum pH of 7.4.

Experimental.

Blood from horses mixed with sodium citrate was centrifuged. The blood corpuscles were washed several times with physiologic salt solution and finally mixed into distilled water. To the haemolyzed solution was added 0.1 M of hydrochloric acid to pH 6.6. The erythrocyte membranes were then flocked. The clear solution was used as enzyme solution.

The pyrophosphatase activity was determined according to a previously described method (SJÖBERG 1940). The reaction constant, K, was calculated. The substance whose effect on the enzyme activity was to be determined, was added to the enzyme solution. The rel. K indicates the relation between the enzyme activity in the presence of an activator or an inhibitor, and the activity under normal conditions.

Experiments to Determine the Identity of the Inhibitory Substance in Blood Plasma.

Twenty-five ml of blood plasma from a horse was dialyzed through collodion membrane against 3 times 25 ml of water. The outside as well as the inside solution was evaporated in vacuum to 25 ml. Two ml of each of the two liquids were added to the reaction mixture. The following values of the enzyme activity were then obtained:

	Rel. K
Enzyme + H_2O	100
» + inside solution	91
» + outside »	37

It is thus evident that the inhibitory effect of the plasma has for the most part been dialyzed.

The outside solution was electrolyzed at pH 6 in an electrolysis vessel partitioned into three parts. Equal amounts of the three fractions were added to the reaction mixture. The following values were then obtained:

	Rel. K
Enzyme + H_2O	100
» + anode solution	79
» + cathode »	10
» + middle »	93

The inhibitory substance had consequently wandered to the cathode and should therefore likely be a positively charged ion. The first thing to think of was then Ca ions. The cathode solution was mixed with ammonium oxalate for precipitation of Ca. With filtrates of this solution the values below were obtained:

	Rel. K
Enzyme + H_2O	100
» + cathode solution	10
» + precipitated cathode solution	77

In another experiment plasma as well as plasma precipitated with ammonium oxalate was added to the reaction mixture giving:

	Rel. K
Enzyme + H_2O	100
» + unmixed plasma	24
» + precipitated plasma	94

The two last experiments show that the inhibition by plasma can be abolished through precipitation of calcium. It is consequently evident that the inhibitory substance consists of Ca ions. The Ca content in blood plasma is about 0.003 M. If about half of this amount is calculated to be ionized, the Ca^{2+} content will be 0.0015 M. In the following it will be shown that this concentration causes practically a 100 per cent inhibition of the pyrophosphatase activity.

Experiments with Inorganic Activators and Inhibitors.

Figs. 1—3 show the inactivation of the pyrophosphatase activity in the presence of a number of anions and cations. Table 1 gives the concentrations of the inhibitors at a 50 per cent inactivation. The most marked inactivation was obtained with Ca^{2+}, Hg^{2+}, Cu^{2+} and F^-. All the determinations were carried out in the presence of optimal amounts of Mg ions.

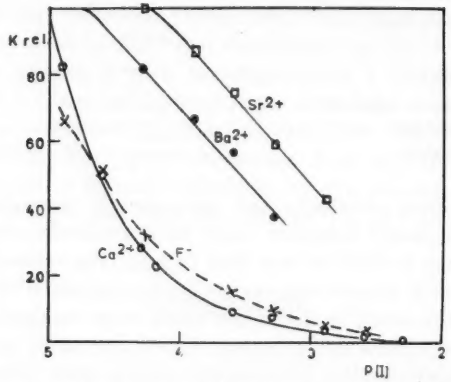


Fig. 1. Inhibition of pyrophosphatase by Ca^{2+}, Sr^{2+}, Ba^{2+}, and F^-. P[I] = — log M inhibitor.

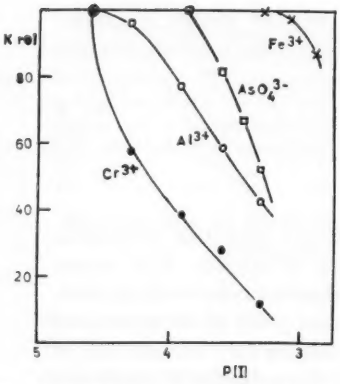


Fig. 2. Inhibition of pyrophosphatase by Fe^{3+}, Al^{3+}, Cr^{3+}, and AsO_4^{3-}.

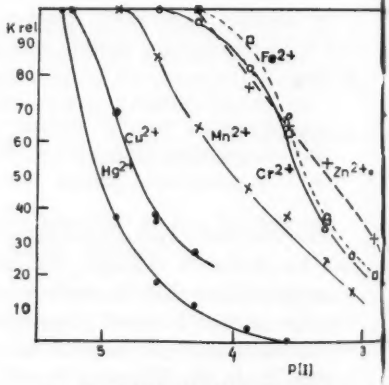


Fig. 3. Inhibition of pyrophosphatase by Hg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, and Cr^{2+}.

Table 1.

Mol Concentration in 50 per cent Inactivation.

Ion	— log Mol	Mol
Hg^{2+}	5.05	0.0000089
Cu^{2+}	4.75	0.000018
Ca^{2+}	4.625	0.000024
Cr^{3+}	4.15	0.000071
Mn^{2+}	4.0	0.00010
Ba^{2+}	3.55	0.00028
Co^{2+}	3.5	0.00032
Fe^{2+}	3.45	0.00036
Al^{3+}	3.45	0.00036
Zn^{2+}	3.3	0.00050
Sr^{2+}	3.075	0.00084
Fe^{3+}	< 2.7	> 0.002
Tl^{3+}	< 2.5	> 0.003
F^-	4.6	0.000025
AsO_4^{3-}	3.3	0.00050

In a couple of experiments the effect of the substrate concentrations on the enzyme activity was investigated. In Fig. 4 the — log substrate concentration is plotted against the reaction constant as well as against mg of split P. These experiments show that increased substrate concentration inhibits the activity, which is a common circumstance. A maximal value of the reaction constant was obtained in a concentration of 10^{-3} M pyrophosphate.

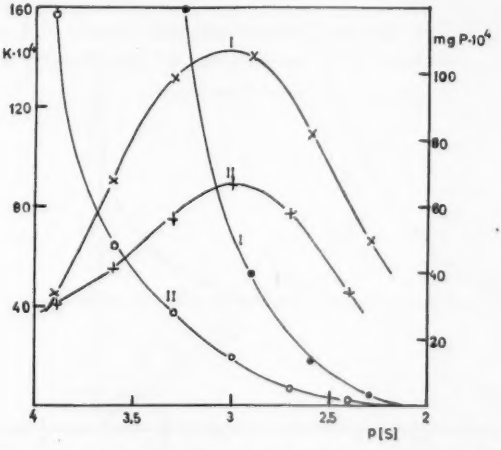


Fig. 4. Enzyme activity and substrate effect. P[S] = — log M substrate concentration as function of rel. K (circles) and Mg hydrolysed P (cross).

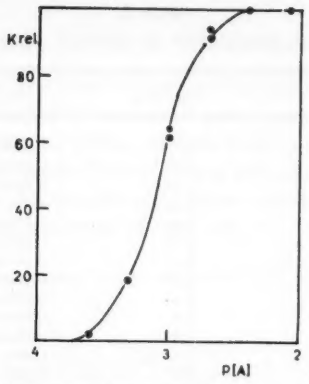


Fig. 5. Magnesium activation curve. P[A] = — log M activator.

Activation of the enzyme activity was obtained with certain substances. Among these were, as is previously shown, Mg^{2+}, which reaches its maximum in a concentration of 0.004 M (p[S] = 2.4) (Fig. 5).

An activating effect was also produced by CN^- and S^{2-} (SH^-) (Fig. 6). A maximal effect of the cyanide ion was obtained at

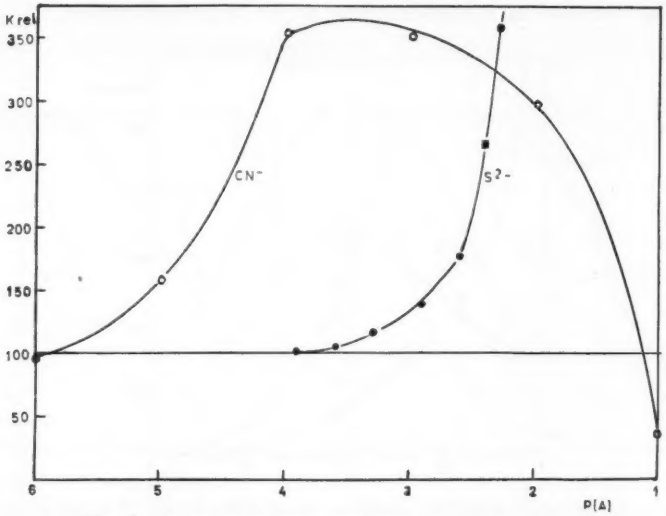


Fig. 6. Activation of pyrophosphatase by CN^- and S^{2-}.

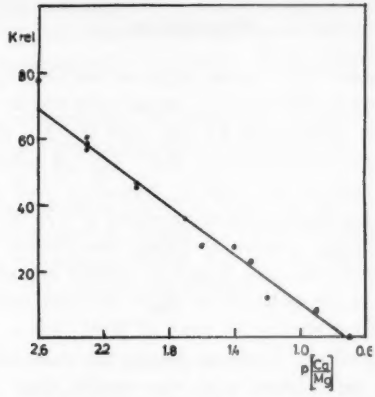


Fig. 7. Antagonism of Mg^{2+} with Ca^{2+}.

10^{-3} — 10^{-4} M. In higher concentrations an inhibitory effect was obtained.

A question of interest is whether an increased Mg^{2+} content can abolish the inactivating effect of other ions, especially Ca^{2+}. In Fig. 7 it is seen that this is the case. However, the affinity to Ca^{2+} is greater. For about 500 times greater Mg^{2+} concentration than Ca^{2+} concentration is required to abolish the inhibitory effect of the latter.

In Fig. 8 it is seen that the enzyme activity within the tested limits are proportional to the enzyme concentration.

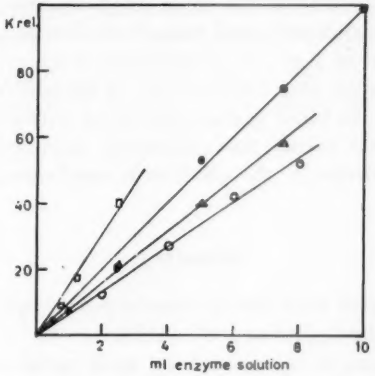


Fig. 8. Enzyme activity with different enzyme concentrations.

Discussion.

Pyrophosphatase from horse erythrocytes belongs to the phosphatases that are inactivated by metal ions which react with SH groups, and also very much by Ca^{2+} and F^-. On the other hand, this phosphatase is activated by Mg^{2+}, CN^- in concentrations less than 0.05 M, and by S^{2-} (SH^-). NAGANNA and MENON (1948) have also found a slight activating effect of CN^- in 0.0002 M concentrations, whereas GORDON (1950) has found an inhibitory effect. This effect was also found in higher concentrations. The action of Ca^{2+} is competitive to Mg^{2+}. In this case Mn^{2+}, which often has an effect similar to that of Mg^{2+}, has a comparatively great inhibitory effect. This contradicts the statements by ROCHE and coworkers but agrees with the results that NAGANNA and MENON (1948) have attained. It is remarkable too that Sr^{2+} and Ba^{2+} act much less inhibiting than does Ca^{2+}. Pyrophosphatase seems to be an enzyme which contains dissociable magnesium which can be exchanged for other metals whereupon the activity is changed. As heavy metal salts have an especially great inhibitory effect, the SH groups also seem to be of importance. Also arsenite, which is believed to react with SH groups, is inhibitory.

Pyrophosphatase is found inside the red blood corpuscles and has no effect on a substrate as long as the blood corpuscles are intact. Not until the blood corpuscles have been haemolyzed and the enzyme has entered the solution is an effect obtained *in vitro*. *In vivo* the Ca^{2+} concentration in the blood plasma is high enough for complete inhibition of the effect of the enzyme, if destruction of the blood corpuscles takes place, even in the presence of an optimal amount of Mg^{2+}. It is reasonable to believe that this is a protective measure and that the rôle of the enzyme is localized completely to the inner parts of the blood corpuscles. Here pyrophosphates are found. And, therefore, it is evident that the enzyme participates in the phosphate metabolism in the blood corpuscle.

Summary.

1. Erythrocytes from horses contain pyrophophatase which is activated by Mg^{2+}, CN^- and S^{2-} (SH^-).

2. The enzyme is inactivated by most metal ions, especially Hg^{2+}, Cu^{2+}, Ca^{2+} and F^-.

3. Ca^{2+} acts as a competitive inhibitor against Mg^{2+}.

4. In blood plasma there is a substance which very much inhibits the pyrophosphatase activity. This substance proved to be Ca^{2+}.

5. Higher concentrations of substrate, Na pyrophosphate, acts inhibiting on the enzyme activity. Maximal effect is obtained in a concentration of 10^{-3} M.

6. The inhibition with heavy metal ions indicates that the enzyme contains SH groups. It seems also to consist of an easily dissociable Mg compound in which Mg can be exchanged for other metals causing the activity to disappear.

References.

Bailey, K., Biochem. J. 1942. *36*. 121.
Bauer, E., Z. physiol. Chem. 1936. *239*. 195.
Csapó, A., Acta physiol. scand. 1949. *19*. 100.
Engelhardt, W. A., M. N. Ljubimova and R. A. Meitina, C. R. acad. sci. U. R. S. S. 1941. *30*. 664.
Fleury, P. and J. Courtois, Enzymologia 1937. *1*. 377, 1938. *5*. 354.
Gergely, J., J. Biol. Chem. 1953. *200*. 543.
Gordon, J. J., Biochem. J. 1950. *46*. 96.
Heppel, L. A. and R. J. Hilmoe, J. Biol. Chem. 1951. *192*. 87.
Kielly, W. W. and O. Meyerhof, Ibidem 1948. *174*. 387.
Meyerhof, O. and P. Olhmeyer, Ibidem 1952. *195*. 11.
Mommaets, W. and K. Seraidarian, J. Gen. Physiol. 1947. *30*. 401.
Naganna, B. and N. Menon, J. Biol. Chem. 1948. *174*. 501.
Roche, J. and Naguyen-van-Thoai, Bull. soc. chim. biol., Paris 1943. *25*. 1365.
— — and M. Roger, Ibidem 1944. *26*. 1047.
Singer, T. P. and E. S. G. Barron, Proc. Soc. Exp. Biol. N. Y. 1944. *56*. 120.
Sjöberg, K., Acta physiol. scand. 1940. *1*. 220.
Spicer, S. S. and W. J. Bowen, J. Biol. Chem. 1951. *188*. 741.
Swansson, M. A., Ibidem 1952. *194*. 685.
Uzawa, S., J. Biochem. Tokyo 1936. *15*. 19.
Ziff, M., J. Biol. Chem. 1944. *153*. 25.

From the Institute of Neurophysiology, University of Copenhagen.

Action Potential Parameters in Normal Human Muscle and their Dependence on Physical Variables.

By

FRITZ BUCHTHAL, CHRISTIAN GULD and POUL ROSENFALCK.

Received 4 May 1954.

Recording of the individual action potential by local random leads within a muscle during slight voluntary contraction reveals a wide variety in duration, amplitude and shape of the electrical response. If the electrical response of the muscle is to be characterized by the mean values of these three parameters, it seemed desirable to investigate their reproducibility and the influence of the amplifier, different types of electrodes and electrode position on duration, amplitude and shape of the muscle action potentials.

Method.

The measurements were performed on the brachial biceps of 13 different subjects, 11 aged 20—22, one 30 and one 45 years of age. For obtaining mean values of action potential duration and amplitude, the electrodes were inserted at random into the muscle and twenty to forty different points were investigated in each examination. For the various points the electrodes were displaced in steps of at least 3 mm in order to ensure that the potentials picked up by the electrode originate from different fibres. Potentials could be led off simultaneously from three points of the same muscle with three electrodes each connected to a differential amplifier and an oscilloscope.

Three types of electrodes were used:

1. *The unipolar electrode* consisted of an insulated steel wire of 0.8 mm diameter ground to a pointed tip. The insulation was removed from

the tip leaving a circular electrode area of 25 to 100 μ in diameter. The electrode impedance averaged 0.5 $\underline{/-45°}$ MΩ measured at 150 c. p. s. with a measuring voltage of less than 10 mV. The unipolar electrode was connected to one grid of the input of the differential amplifier. The insulated steel wire was introduced through a subcutaneously inserted cannula into the muscle. The cannula served as reference electrode. A flexible metal plate electrode (5 × 10 cm) was connected to the earth terminal of the amplifier. It was placed near the muscle under examination and prevented electrocardiographic artifact.

2. *The concentric electrode,* unless otherwise stated, consisted of a cannula with an external diameter of 0.7 mm. Inside this cannula a platinum wire, 0.1 mm in diameter, was introduced insulated from the metal cannula by means of a thin glass tubing (BUCHTHAL 1944). The open space within the cannula was filled up with an insulating cement. The tip of the cannula was ground to an angle of 15—20 degrees. Thereby, the area of the internal electrode became elliptic with diameters of approximately 100 and 400 μ. The impedance averaged 50,000 $\underline{/-45°}$ Ω measured at 150 c. p. s. with a measuring voltage of less than 10 mV. In order to utilize the differential amplification the centre electrode was connected to one grid and the cannula as reference electrode to the other grid of the input valves. The subject was connected to the earth terminal of the amplifier by a large plate electrode.

3. *The bipolar electrode* was constructed as was the concentric but with two insulated platinum wires within the cannula. The centres of the leading-off areas were situated on the long axis of the elliptic surface of the tip. The dimensions of the leading-off areas and the impedance of each individual electrode were the same as for the concentric electrode. The distance between the centers of the leading-off areas of the two cores was 0.52 mm. Each core was connected to a grid, and the cannula was either connected to earth, or was free and a large plate electrode served as earth connection.

Amplifiers:

The amplifiers were push-pull throughout with large common cathode resistors and RC coupling. The noise level was less than 2 μV r. m. s. It was measured by adjusting a sinusoidal input voltage so that the amplifier output voltage exceeded the noise level by 3 db. The blocking time of the amplifier, *i. e.* its recovery time after an overload, was about one second. The input impedance of the amplifier was 100 MΩ in parallel with 100 $\mu\mu$F. The voltage division between the electrode impedance and the impedance of the amplifier can be of essential influence on the effective frequency response. This was measured as illustrated in Fig. 1. The signal from a generator was led through a low impedance plate electrode and was recorded by a *different* electrode. Both electrodes were placed in a 0.9 per cent sodium chloride solution. The same arrangement was used for the measurement of differential amplification.

The differential amplification (in phase signal rejection) is defined as the amount by which a voltage *common* to both inputs must exceed a

voltage *between* the two input terminals in order that both voltages shall give the same output. It exceeded 3,000 over the entire frequency range of the amplifier. However, the transmission from electrode to amplifier seriously affects the overall differential amplification. This is often neglected when differential amplifications as high as 10^4 to 10^5 times are claimed. The actual differential amplification under working conditions is increased considerably by the use of a high input impedance. For example measuring with the arrangement shown in Fig. 1 with the

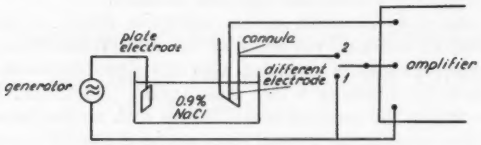


Fig. 1. Set-up for measuring the frequency band (1) and differential amplification (2) of amplifier plus recording electrode.

cannula as reference electrode in the 0.9 per cent sodium chloride solution, a differential amplification of more than a hundred times was obtained over the entire frequency range transmitted: 2 to 10^4 c. p. s. This ensures that the potential difference between the *different* electrode and the reference electrode is not distorted by potentials common to these electrodes, as long as the common potential does not exceed the potential to be measured. With the bipolar electrode a differential amplification of more than 100 was found over the entire frequency range as well, making it possible to measure the actual potential difference between the two internal electrodes.

Recording:

Action potentials were recorded photographically from three 1.5″ cathode ray oscilloscopes. The sweep velocity normally used was 100 cm per second. With the paper speed of 5 cm per second the X-axis deviated only slightly from the horizontal line. The sweep frequency of 5 c. p. s. prevented overriding of the individual sweeps. A separate set of three oscilloscopes was used for visualisation (DISA Electromyograph). Each channel could be connected to a loudspeaker.

Definition of Action Potential Parameters.

A. Action potential duration may be measured either as total duration (*e. g.* BUCHTHAL and CLEMMESEN 1941, PETERSÉN and KUGELBERG 1949) or as duration of the so-called "negative spike of the triphasic potential" (JASPER and BALLEM 1949, LUNDERVOLD and CHOH-LUH LI 1953). For reasons mentioned below, *total duration* was measured in the present study.

Action potentials from isolated frog muscle fibres were recorded in a volume conductor and were found to be mainly diphasic with the first positive phase of a similar amplitude as the following negative. A final positive deflection disappeared in most cases in the noise level (Fig. 2). The active region of the membrane is represented by the monophasic potential recorded in air.

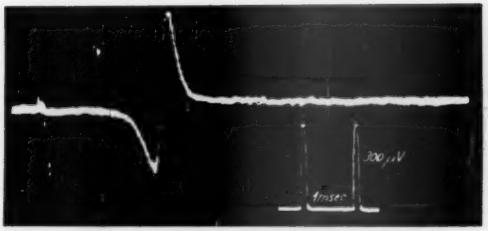


Fig. 2. Single muscle fibre potential from frog's semitendinosus, recorded in Ringer's solution. 20 μ distance between fibre and electrode (diameter 50 μ). 20° C.

The triphasic potential obtained in a volume conductor has at the surface of the fibre the same duration as the monophasic potential (LORENTE DE NÓ 1947). Therefore, the negative spike of the action potential recorded in a volume conductor cannot be considered to represent the total duration of the membrane changes. Conditions are still more complex for the motor unit potential which is the result of a summation of potentials from many fibres with differences in start and propagation. Hence, the total duration of the muscle action potential corresponds to the membrane changes, and positive deflections arising from volume conduction cannot properly be called derivation artifacts (JASPER and BALLEM 1949).

When the beginning and ending of the potential were abrupt, the total duration could be measured without difficulty. However, disturbances from other sources can introduce uncertainties when the onset is gradual or the action potential has a "tail". These disturbances may be attributable to more distant motor units, or, with unipolar electrodes of high impedance (> 1 MΩ at 150 c. p. s.) to an electrode noise exceeding that of the amplifier input stage. These disturbances were evaluated by recording each action potential from the individual points at least three times, five to ten times in the case of potentials with low amplitude. Potentials

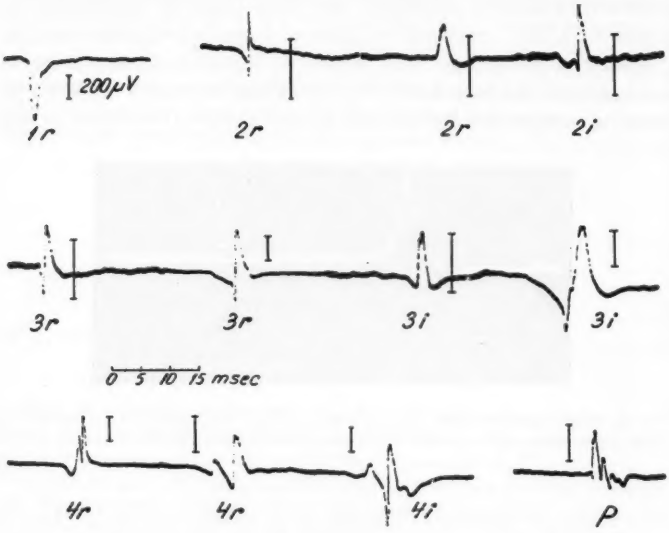


Fig. 3. Samples of different shapes of action potentials.
The numbers below the action potentials indicate the number of phases.
r = regular
i = irregular
p = polyphasic

of less than 50 μV were not included in the measurements. The action potential was recorded as maximum deflection on the screen in order to prevent the loss of low amplitude components for the determination of duration.

B. The amplitude of the action potential was measured for all potentials whose duration could be determined. It was defined as the peak to peak deflection, *i. e.* the difference between the highest positive and negative value of the potential. Except with action potentials of the lowest amplitude, noise arising from other motor units gives less uncertainty than in the determination of action potential duration. No particular search was made for the maximum amplitude (JASPER and BALLEM 1949, LUNDERVOLD and CHOH-LUH LI 1953).

C. The shape of the action potential was characterized by the number of deflections across the base line. According to the number of phases the potential was denoted as monophasic, diphasic, triphasic, tetraphasic and polyphasic. The letter comprises all potentials with five phases or more. Examples of the different potential shapes are given in Fig. 3.

Statistical Treatment of the Results.

The action potential durations and amplitudes are represented in histograms (percentage distribution curves). The arithmetical mean duration is the sum of the action potential durations divided by their number (N). The graphical is the 50 percentile mean duration. The standard deviation is defined as

$$SD = \sqrt{\frac{\Sigma\, d^2}{N - 1}} \qquad (1),$$

where d denotes the deviation of the individual value from the arithmetical mean duration.

From the mean error SE, defined by

$$SE = \frac{SD}{\sqrt{N}} \qquad (2),$$

a measure of the statistical significance of a difference in action potential duration (η) is obtained as

$$\eta = \frac{t'_m - t_m}{\sqrt{SE'^2 + SE^2}} \qquad (3)$$

where t'_m and t_m are mean durations whose difference is under investigation, SE′ and SE the corresponding mean errors.

A Gaussian distribution can be applied, since the number of degrees of freedom is always more than forty. Table 1 gives the probabilities that a difference between two mean values is real.

Table 1.

Measure of statistical significance.

η	Probability in per cent that the difference is real
1	68
2	95.4
2.6	99
3.3	99.9

Results.

Impedance and frequency band.

The distortion of the recorded action potentials depends on the electrode impedance, the frequency response of the amplifier, its input impedance and the degree of differential amplification.

The impedance of the electrodes was determined as a resistance in parallel with a capacitance by means of a bridge. The impedance decreases essentially with increasing measuring voltage. In Table 2 an example is given for the impedance of a concentric electrode as a function of the voltage. An abrupt decrease in impedance occurred with a measuring voltage exceeding 100 mV. When the measurements were repeated after a preceding measurement with high voltage, the low impedance of the electrode was maintained until the electrode surface was changed again by drying or by other manipulations. In view of these results it was considered necessary in the impedance measurements to apply voltages which were of the same order of magnitude as the action potentials (< 10 mV).

Table 2.

Impedance of a concentric electrode as a function of measuring voltage at 800 c. p. s. in 0.9 per cent NaCl solution.

Measuring voltage (volt)	Impedance	
	Z $10^3\ \Omega$	angle degrees
0.05	27	— 68
0.2	20	— 67
0.5	6.5	— 50
2.0	1.7	— 20
5.0	1.2	— 12
10	0.9	— 8
0.05	1.9	— 38
0.05[1]	11	— 60

[1] After drying.

As a function of the measuring frequency the electrode impedance varied for different concentric electrodes between $290 \cdot 10^3$ $\underline{/-\ 60°}\ \Omega$ at 20 c.p.s. and $7 \cdot 10^3$ $\underline{/-\ 41°}\ \Omega$ at 5,000 c.p.s.; for unipolar electrodes between 10^6 $\underline{/-\ 35°}\ \Omega$ at 20 c. p. s. and $62 \cdot 10^3$ $\underline{/-\ 45°}\ \Omega$ at 5,000 c. p. s. A frequency of 150 c. p. s. was chosen to

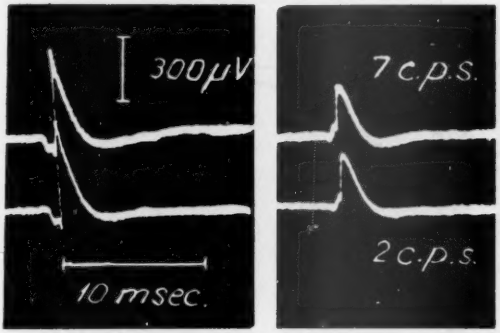


Fig. 4. Two examples for the distortion of the terminal portion of the muscle action potential produced by too high a lower limiting frequency of the amplifier. Negative deflection upwards; lower limiting frequency for the upper curves 7 c. p. s.; for the lower curves 2 c. p. s.

compare the impedance of the different electrodes used in the present investigation in order to approach conditions actually present in muscle.

The effect on the action potential of the *upper and lower limiting frequency*, f_u and f_l, defined by 3 db discrimination, was determined by recording the same action potential at various limiting frequencies. On one beam of the oscilloscope the potential was photographed ten times at each set of limiting frequencies and on the other, simultaneously, at the widest frequency band.

The *lower* limiting frequency was varied from one to one hundred c. p. s. For f_l equal seven c. p. s., a distortion may occur at the end of the action potential causing a change in both amplitude and duration (Fig. 4). From experiments on potentials of various shapes and durations a lower limiting frequency of 2 c. p. s. was found to be requisite. A lower limiting frequency of 30 c. p. s. as recommended by BULLER and STYLES (1952) introduces serious distortions.

An *upper limiting frequency* of 2,000 c. p. s. was satisfactory for recording total durations down to one msec. However, this upper limiting frequency distorts the amplitude of the potentials, the degree of distortion depending on the steepness of the potential change. For a potential change 100 μsec. in duration there was a five per cent decrease in amplitude, when the upper limiting frequency was decreased from 25,000 to 10,000 c. p. s. (Fig. 5),

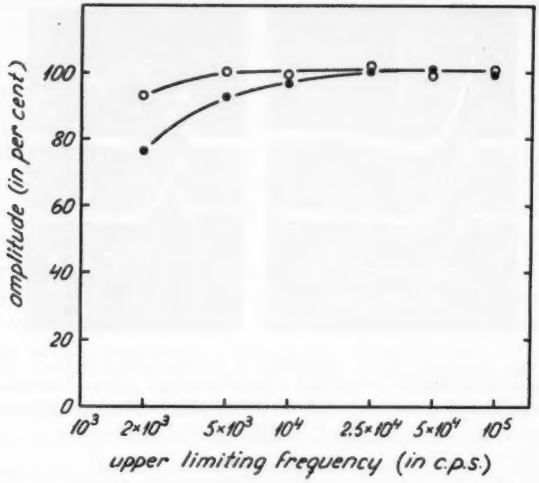


Fig. 5. Action potential amplitude as a function of the upper limiting frequency of the amplifier. The amplitude is expressed in per cent of its value at 10^6 c.p.s.

	duration (msec.) total	steep deflection
○———○	19.0	0.4
●———●	2.7	0.1

for a potential change 300—500 μsec. in duration the decrease occurred at 4,000 c. p. s. The upper limiting frequency is still more critical for the determination of the duration of the steep potential change. With a decrease in the upper limiting frequency from 100,000 to 25,000 c. p. s., a potential change of a 100 μsec. duration is recorded as 150 μsec.

Obviously the frequency characteristic of an amplifier must be chosen, according to what parameter of the action potential is to be measured. It is important that it be wide enough to allow an undistorted recording, though not wider than necessary, since the noise level increases with the square root of the frequency band. In the present study a lower limiting frequency of two c. p. s. and an upper limiting frequency of 10,000 c. p. s. were used.

The *input impedance* of the amplifier with electrode cable was 100 MΩ in parallel with 100 $\mu\mu$F. At this value the transmission link consisting of one of our electrodes and the input impedance transmits at least the same frequency band as the amplifier. At

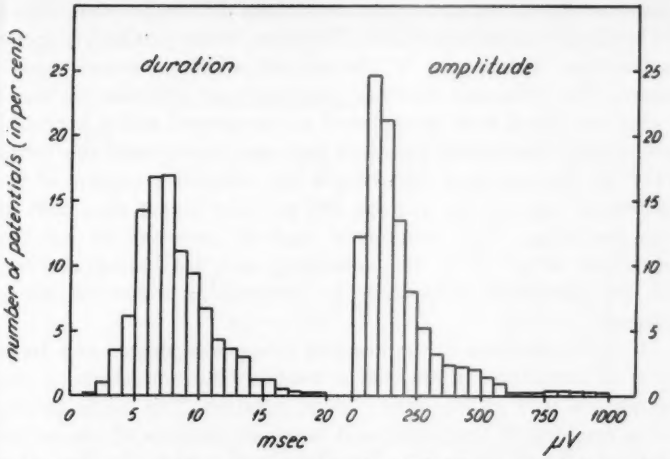


Fig. 6. Histograms of duration and amplitude. 1,268 different potentials recorded with concentric electrodes from the brachial biceps of J. W., 21 years of age.

the lower frequency of 2. c. p. s. and with a numerical value of the input impedance of 100 MΩ, the maximum electrode impedance which can be used is 20 MΩ. At the upper limiting frequency of 10,000 c. p. s. and with the input capacitance of 100 $\mu\mu$F the maximum electrode impedance which can be used is 50,000 Ω.

Distribution curves for action potential duration and amplitude.

In Fig. 6, histograms are given of action potential duration and amplitude obtained with 12 different concentric electrodes in 25 examinations of the brachial biceps of a normal subject, 21 years of age. Durations and amplitudes varied within rather wide limits. The standard deviation for duration amounted to approximately 30 per cent of the mean duration (7.91 msec., 1,268 potentials) and the standard deviation for amplitude to 60 per cent of the mean amplitude (202 μV).

The histograms of both duration and amplitude were asymmetrical. This implies that the graphical mean value deviates from the arithmetical. In the histograms of action potential duration (Fig. 6), the graphical mean value was an average of five per cent lower than the arithmetical. The standard deviation of the descending part of the histogram averaged 60 per cent higher than that of the ascending part. For the statistical evaluations based on

standard deviation and mean error these differences were found to be of only minor importance. Therefore, it was justified to apply a Gaussian distribution in the estimation of statistical significance. The difference between graphical and arithmetical mean value was much more pronounced in the case of action potential amplitude, the former being 26 per cent lower than the latter (Fig. 6). The standard deviation of the descending portion of the histogram was on the average 190 per cent higher than that of the ascending. This asymmetry made it necessary to use the standard deviation of the ascending and descending portions of the histogram separately in determining statistical significance.

If mean duration in the brachial biceps was plotted as a function of amplitude, there was a tendency for potentials of low amplitude (100 μV) to have a short duration. This might be due to a masking of the initial and terminal portions of the action potential by noise. In the anterior tibial muscle, on the other hand, the correlation was clearer: potentials with an amplitude below 200 μV were 20 per cent shorter than potentials with an amplitude above 300 μV.

Reproducibility of mean action potential duration and amplitude.

The reproducibility of mean duration and amplitude was tested in 20 separate examinations of a left brachial biceps of a normal subject. For each examination 20 action potentials were led off, all with the same concentric electrode. Only one of the twenty mean durations deviated from the over-all mean duration by more than twice the mean error (Fig. 7). This indicates that the mean duration was reproducible from one examination to the other. Four of the twenty mean amplitudes deviated from the over-all mean value by more than twice the mean error. This is 45 per cent above the variation which might be expected statistically. Taking the asymmetry of the histograms into account the variation is still 25 per cent too high (Fig. 7).

Mean duration and amplitude with electrodes of different types and dimensions.

Electrodes of different type: Unipolar electrodes and concentric electrodes of approximately equal impedance (*i. e.* leading-off area) gave values for duration identical within the experimental error:

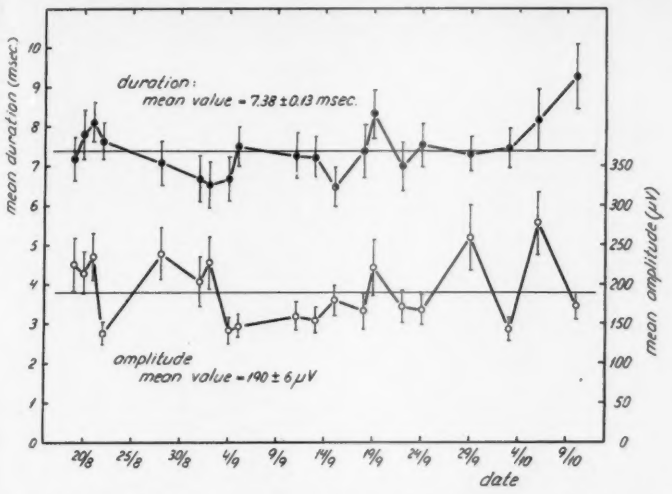


Fig. 7. Reproducibility of mean duration (left ordinate) and amplitude (right ordinate). An average of 20 potentials recorded on different days with the same concentric electrode in the brachial biceps of J. W., 21 years of age. The vertical lines denote the mean error.

7.81 ± 0.17 msec., 226 potentials; and 7.77 ± 0.08 msec., 1,048 potentials, respectively (Fig. 8). Previously, the mean duration obtained with the unipolar electrode was 14 per cent higher than that of the concentric electrode (GULD 1951). The present finding of no difference might be due to the shorter distance between the unipolar and the reference electrode in this as compared with the previous study. This is discussed further in the section on the effect of the cannula.

Also different in the present experiments as compared with those previously reported (GULD 1951, BUCHTHAL and PINELLI 1952) is the 35—55 per cent higher mean duration. This is due to the improved recording technique which allows recognition of the onset and the tail of the potential with greater certainty than previously.

The *mean duration* measured with eleven concentric electrodes of the same batch (100 potentials with each) varied between 7.17 and 8.95 msec. (Fig. 8). There was no systematic variation with the outer diameter of the cannula within the range 0.38—0.90 mm. Nor was there a systematic variation with the obliqueness

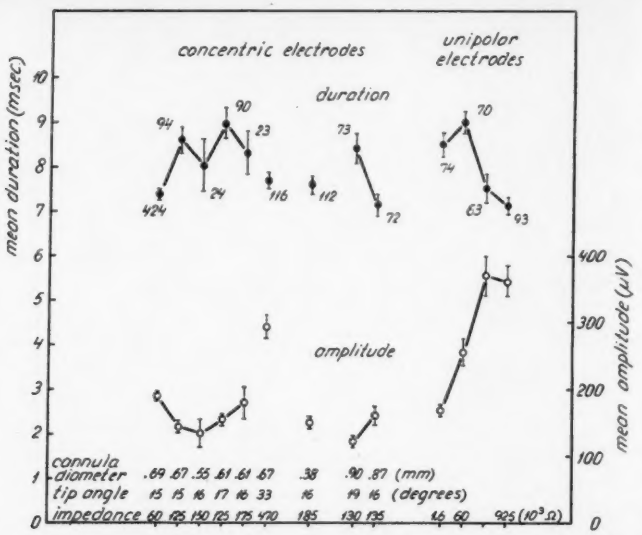


Fig. 8. Mean duration (left ordinate) and amplitude (right ordinate) for different concentric and unipolar electrodes. The figures on the upper curves denote the number of potentials recorded with each electrode. Vertical lines indicate mean errors. Dimensions and impedance (at 150 c. p. s.) are given on the figure.

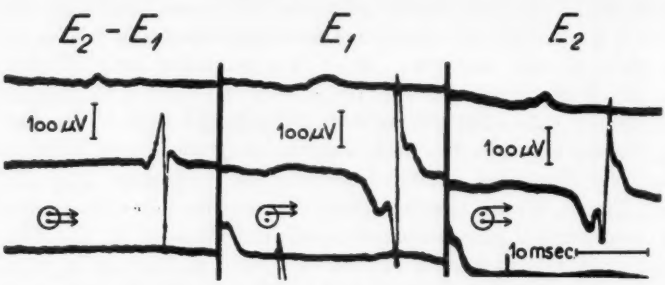
of the electrode tip (in the range 15—33°) as reported by PETERSÉN and KUGELBERG (1949). This implies that the leading-off area of the central core is not crucial for the determination of the mean duration for areas varying between $3 \cdot 10^4$ and $6 \cdot 10^4$ μ^2, with impedances of $6 \cdot 10^4$ $\angle -57°$ Ω to 0.47 $\angle -71°$ MΩ measured at 150 c. p. s.

The variation in mean duration for electrodes of the same batch cannot be explained by statistical fluctuations due to the limited number of action potentials recorded, but must be ascribed to differences inherent in the electrodes. This is supported by the finding that the mean durations determined with different electrodes of the same batch showed consistent variations on three different subjects.

The *mean amplitudes* obtained with different concentric electrodes did not depend on the diameter of the cannula or the leading-off area of the inner core (Fig. 8). A decrease in the angle between the oblique surface and the axis of the cannula from 33° to 15° was, however, associated with a decrease in mean ampli-

tude of the recorded action potentials. The mean amplitude was 169 ± 5 μV (922 potentials) for concentric electrodes with a tip angle of 15—20°, and 294 ± 18 μV (116 potentials) for a tip angle of 33°. High amplitudes were found with the cores of the bipolar electrodes (250—350 μV) as well, although the angle of the obliquety was only 20°. The mean amplitudes obtained with different *unipolar* electrodes exhibited considerable variations (170—370 μV, Fig. 8). Unlike the concentric, the unipolar electrodes showed a dependence of mean amplitude on the impedance of the electrode, the amplitude increasing 120 per cent with an increase in impedance from 5,000 Ω, 590 $\mu\mu$F, to 1.7 M Ω, 0.9 $\mu\mu$F. The high mean amplitude found with the high impedance unipolar electrodes is due to the smaller leading-off areas as compared with concentric electrodes. The electrode records a mean value of the potential which is spread over the leading-off area. The smaller the leading-off area, the smaller the amplitude variation over it and the higher the amplitude. Another possible factor determining the high mean amplitude is that the high impedance electrodes give rise to an increase in noise level, possibly masking action potentials of small amplitude.

The bipolar electrode: As has been reported previously (PINELLI 1949, LANDAU 1951, and LUNDERVOLD and CHOH-LUH LI 1953) the bipolar and the concentric methods of leading-off were compared by simultaneous recording of the potential difference between each of the inner cores of the bipolar electrode and the cannula, and of the potential difference between the cores (Fig. 9). The mean action potential duration obtained with bipolar recording was in our experiments 25 per cent shorter than with concentric recording. This is due to a partial cancelling of the initial and terminal portions of the potentials picked up by each of the inner cores (*differentiation*). Each of the inner cores relative to the cannula gave identical mean durations. The amplitudes recorded between each core and the cannula were not identical. In general, one showed a considerably higher amplitude than the other, probably due to different spatial relations with respect to the active fibres. The amplitude at bipolar recording was always close to the highest amplitude simultaneously recorded between one of the cores and the cannula. Thus, the mean amplitude with bipolar was always higher than the mean amplitude with concentric recording (Fig. 9).



Type of recording	Mean duration (msec.)	Mean amplitude (μV)
Core 1 to cannula (E_1)	8.47 ± 0.22	249 ± 17
Core 2 to cannula (E_2)	8.38 ± 0.22	326 ± 21
Core 1 to core 2 ($E_1 - E_2$)	6.31 ± 0.20	371 ± 20

120 potentials from a normal brachial biceps.

Fig. 9. Recording between the cores (E_2—E_1) and between cores and cannula (E_1 and E_2) of a bipolar electrode.

With shorter distances (0.2 mm) between the cores than were used here (0.5 mm), a differentiation of the amplitude occurs as well and, therefore, the mean amplitudes were lower with bipolar than with concentric electrodes (PETERSÉN and KUGELBERG 1949, LUNDERVOLD and CHOH-LUH LI 1953). Durations are also shorter due to a better differentiation of the initial and the terminal portions of the potential.

With bipolar electrodes, the position of the electrode relative to the direction of the muscle fibres affects the mean values of duration. The mean duration was significantly shorter (25 per cent) when the electrode was inserted parallel to the direction of the fibres (1,080 potentials) than when it was perpendicular to them (2,194 potentials). Since this effect occurs with bipolar electrodes, it cannot be caused by differences in depth of insertion of the cannula (see below). Rather it is an expression of the distribution of the potential field around the activated fibres. The shorter duration with the electrode perpendicular to the longitudinal axis of the fibre indicates that the field strength in the initial and terminal part of the potential is essentially less perpendicular than parallel to the fibre axis.

The influence of the cannula.

Even the cannula of a concentric electrode picked up potentials at slight effort. If the cannula was used as *different* electrode referred to a distant subcutaneous electrode, the mean amplitude of the potentials was 25 to 30 per cent of the amplitude obtained with ordinary unipolar or concentric recording. The potential recorded with a concentric electrode is the difference between that picked up by the core and that on the cannula. Therefore, the potentials recorded between the core and cannula were ten per cent shorter than potentials picked up by either core or cannula referred to a distant subcutaneous electrode. A similar difference was previously found between mean values obtained with unipolar and concentric electrodes. This was due to the larger distance between the *different* unipolar electrode and the cutaneous electrode (GULD 1951). Different properties of the cannula surface may make it more or less apt to pick up potentials which partially compensate the potentials on the core. This might account for the consistent variations in mean action potential duration as obtained with different concentric electrodes of the same batch. Use of a unipolar electrode, *e. g.* the core of a concentric electrode referred to a distant electrode would avoid this variable. On the other hand, the increased pick up of potentials from more distant units with this type of recording introduces a much higher degree of inaccuracy in the determination of action potential duration.

The fact that the cannula of the concentric electrode picks up potentials, can explain the differences in mean duration and amplitude obtained in different depths of the muscle. Both showed a statistically significant increase, the deeper the electrode was inserted into the muscle (Table 3). With increasing depth of the cannula in the muscle, the mean amplitude of the action potential picked up by the cannula of the concentric electrode decreased, since the potential is the average value over the portion of the cannula in contact with the conducting tissue. The decrease was from 90 μV in the superficial portion to 25 μV in the deep portion of the muscle. Hence, the cannula will act more as a truly indifferent electrode, the deeper it is inserted into the muscle, *i. e.* with a deeply inserted cannula the potentials picked up by it will to a lesser degree compensate potentials on the core. This results in the recording of action potentials with a longer duration and a higher

Table 3.

Mean values of action potential duration and amplitude in the superficial layers and in the interior of the brachial biceps (concentric electrodes).

	Number of action potentials	Mean duration (msec.)	Mean amplitude (μV)
Surface	160	7.94 ± 0.24	179 ± 7
Halfway	220	8.66 ± 0.21	160 ± 6
Middle	190	9.15 ± 0.22	319 ± 7

amplitude, as compared with the potentials recorded with the cannula in the superficial layers of the muscle.

The shape of the action potentials.

For the study of the shape, muscle action potentials were led off from the brachial biceps of ten normal subjects, 20 to 22 years of age (Table 4). Di- and triphasic potentials comprised about 80 per cent of all action potentials for all types of electrodes. The incidence of polyphasic potentials was three to five per cent of all potentials.

Table 4.

Distribution of shape of action potentials

(brachial biceps of normal subjects, 20—22 years of age).

Type of electrode	Total number of potentials	Number of potentials (in per cent)				
		Monophasic	Diphasic	Triphasic	Tetraphasic	Polyphasic
Concentric	692	3.2	45.6	41.3	6.5	3.4
Unipolar	619	2.4	30.1	52.4	9.9	5.2
Bipolar	488	3.0	47.5	35.5	11.0	3.0

The mean duration of the action potentials increased with the number of phases, the increase from monophasic to polyphasic potentials amounting to 60 per cent for unipolar and concentric electrodes. The mean amplitude was dependent on the shape of the potential, only in that it was about 30 per cent lower for monophasic potentials.

Summary.

Duration, amplitude and shape of muscle action potentials were recorded during slight voluntary effort. Their dependence on some physical variables was investigated.

The influence of the frequency response of the amplifier on the action potential parameters has been examined. An upper limiting frequency of 10^4 c. p. s. and a lower limiting frequency of two c. p. s. allow a practically undistorted recording of total duration and amplitude of the action potentials down to a duration of one msec. An undistorted recording of the duration of the steep negative deflection of the action potentials (100—300 μsec.) requires an upper limiting frequency of $5 \cdot 10^4$ c. p. s. A lower limiting frequency above two c. p. s. can give rise to a distortion in the last portion of the action potential affecting both duration and amplitude.

The numerical values of the impedance of the recording electrodes used in the present study were $5 \cdot 10^3$ to 10^6 Ω (150 c. p. s.). The input impedance of the amplifier was 100 MΩ in parallel with 100 $\mu\mu$F. With a frequency band of 2 to 10^4 c. p. s. this input impedance allows undistorted recording with electrode impedances up to 20 MΩ at 2 c. p. s. and up to 50,000 Ω at 10^4 c. p. s.

The electrode impedance decreased abruptly when the measuring voltage exceeded 100 mV. Therefore, in order to approach conditions actually present in muscle, impedance measurements were performed with a measuring voltage of 10 mV.

The common mode rejection of the amplifier was 3,000 times and of the amplifier plus electrodes 100 times over the entire frequency range. This excludes disturbances from internal or external in phase signals.

Total duration was used as parameter, since it corresponds to the changes in the membrane of the activated fibres. The gradual onset and the tail of the motor unit potential may be distorted by potentials picked up from more distant motor units. For an accurate determination of the total duration it was, therefore, necessary to record the same action potential three to ten times.

In a single examination of a muscle comprising 20 to 40 different action potentials, the mean duration is determined with a mean error of seven to five per cent and the mean amplitude with a mean error of 20 to 15 per cent.

The mean action potential duration was identical with concentric and unipolar recording. The mean amplitude was higher with a unipolar than with a concentric electrode. In repeated examinations of the same muscle, using the same electrodes, the values of the mean duration were reproducible within the statistical fluctuations, whereas mean amplitude varied forty per cent more than to be expected statistically.

For different electrodes of the same batch the mean duration varied 25 per cent, while for the same electrode the variation only was 3 per cent. This difference could not be accounted for by differences in electrode impedance, leading-off area or obliqueness of the tip. Unlike the concentric, the unipolar electrodes showed an increase in mean amplitude with increasing impedance.

The bipolar electrode recorded a shorter duration than the concentric or the unipolar electrodes, the durations being shorter the smaller the distance between the cores. The reduction in duration is due to a partial cancelling of the initial and the terminal portions of the potentials picked up by each of the inner cores. Similarly, with a concentric electrode potentials picked up by the cannula partially cancel potentials on the core. The mean amplitude of the action potentials picked up by the cannula decreased with increasing depth of insertion. This results in the recording of potentials with longer duration and higher amplitude in the deeper than in the superficial portions of a muscle. With bipolar electrodes the mean duration was 25 per cent shorter when the electrode was inserted parallel to the direction of the muscle fibres than when it was perpendicular to them.

Di- and triphasic potentials comprised about 80 per cent of all potentials. Action potentials with more than four phases (polyphasic potentials) occurred in 3 to 5 per cent of all potentials.

The work was supported by grants from the Michaelsen Foundation and the Rockefeller Foundation.

References.

BUCHTHAL, F., Nord. Med. 1944. *24*. 2245.
BUCHTHAL, F. and Sv. CLEMMESEN, Acta psychiat. Kbh. 1941. *16*. 143.
BUCHTHAL, F. and P. PINELLI, Acta Med. Scand. 1952. *142* Suppl. 266 p. 315.
BULLER, A. J. and P. STYLES, Ann. Phys. Med. 1952. *1*. 37.
GULD, CHR., Acta Physiol. Scand. 1951. *25*. Suppl. 89. p. 30.
JASPER, H. and G. BALLEM, J. Neurophysiol. 1949. *12*. 231.
LANDAU, W. M., EEG Clin. Neurophysiol. 1951. *3*. 163.
LORENTE DE NÓ, R., Studies from the Rockefeller Inst. for Med. Res. 1947. *132*. Chapter XVI.
LUNDERVOLD, A. and CHOH-LUH LI, Acta psychiat. Kbh. 1953. *28*. 201.
PETERSÉN, J. and E. KUGELBERG, J. Neurol. 1949. *12*. 124.
PINELLI, P., C. R. IV. Congr. Neurol. Int., Paris 1949. *3*. 98.

(From the Institute of Neurophysiology, University of Copenhagen).

Action Potential Parameters in Normal Human Muscle and their Physiological Determinants.

By

FRITZ BUCHTHAL, P. PINELLI[1] and P. ROSENFALCK.

Received 11 May 1954.

In the preceding paper (BUCHTHAL et al. 1954 a) physical factors have been dealt with of importance for the determination of mean values of action potential duration, amplitude and shape. The present study concerns the influence of some physiological factors on these mean values. The effect of age, temperature, degree of contraction and fatigue have been investigated.

Method.

Material: The influence of age was studied on the left brachial biceps of 130 subjects between 1 and 85 years of age. The other physiological variables were examined on the brachial biceps of 10 normal subjects 20 to 22 years of age, seven females and three males.

Temperature control: In experiments with varying intramuscular temperature, the brachial biceps was cooled by two ice-bags, applied to the volar surface of the upper arm. In these and in all other experiments, temperature was controlled by a thermocouple inserted into the muscle (BUCHTHAL et al. 1944). In the standard experiments, the temperature was kept constant at 36.5° C. When the temperature was lower, it was adjusted to this value by applying a heating pad to the upper arm.

Electrodes: The muscle action potentials were led off simultaneously from three regions of the same muscle with three electrodes each connected to a differential amplifier and an oscilloscope. The electrodes were

[1] Working with a fellowship from the Rask-Ørsted Foundation.

inserted at random into the muscle and the mean value of duration and amplitude for each muscle was determined from potentials led off from twenty to forty different points.

Concentric electrodes with leading-off areas of $3—6 \cdot 10^4 \mu^2$ and bipolar electrodes with a distance between the leading-off areas of 0.5 mm were used. The impedance averaged $50{,}000 \angle -45\,\Omega$ measured at 150 c. p. s. with a measuring voltage of less than 10 mV.

Amplifiers: The amplifiers had a lower limiting frequency of 2 c. p. s. and an upper limiting frequency of 10^4 c. p. s., defined by 3 db discrimination. The input impedance was 100 $M\Omega$ in parallel with 100 $\mu\mu$F. The differential amplification (in phase signal rejection) of amplifier plus electrode was more than a hundred over the entire frequency range of the amplifier. The noise level of the amplifiers was less than 2 μV r. m. s.

Recording: The action potentials were recorded photographically from three 1.5″ cathode ray oscilloscopes. Normally a sweep velocity of 100 cm per sec. and a sweep frequency of 5 c. p. s. were used. The paper speed was 5 cm per sec.

Measurement: Definition of the action potential parameters and the statistical treatment of the results have been described in the preceding paper (BUCHTHAL et al. 1954 a).

Results.

1. Mean duration, amplitude and shape as a function of age.

In preliminary investigations, BUCHTHAL and PINELLI (1951, 1952) found the mean action potential duration to increase with age over the range two to seventy years. This has been corroborated in the brachial biceps of 130 normal subjects, 88 below 15 and 12 above 40 years of age (Fig. 1). For the age group 0—4 years a mean duration of 5.7 msec. was found. At 70—80 years of age the mean duration was 10.0 msec., *i. e.* 75 per cent higher than for the youngest group. The standard deviation of the mean durations for different subjects of the same age group increased as well. At 0—4 years it amounted to 0.5 msec., whereas in the older group it was 1 msec.

The mean amplitude of the muscle action potentials was independent of the age of the subject and amounted to 192 ± 2 μV for the total material. The standard deviation for the mean amplitudes of different subjects was 60 μV.

The distribution of the action potentials according to their shape was different in different age groups (Table 1). In children below four years of age, triphasic potentials were of relatively more

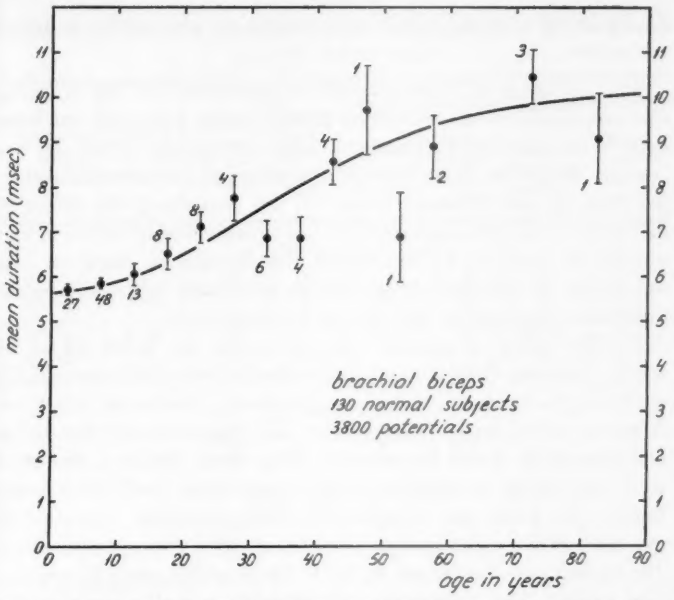


Fig. 1. Mean action potential duration as a function of age. The vertical lines denote mean error, the figures on the curve, number of subjects. Concentric electrodes, brachial biceps.

Table 1.

Distribution of shape of action potentials for different age groups.

(Brachial biceps; concentric electrodes.)

Age (years)	Total number of potentials	Number of potentials (in per cent)				
		Monophasic	Diphasic	Triphasic	Tetraphasic	Polyphasic
0— 4	170	2.0	31.5	56.5	5.5	4.5
20—22	692	3.2	45.6	41.3	6.5	3.4

frequent occurrence than diphasic. In adults (20—22 years of age), triphasic and diphasic potentials were equally represented.

2. Influence of sex.

No statistically significant differences have been found between mean durations and amplitudes in males as compared with females.

3. Effect of intramuscular temperature on the action potential parameters.

The influence of intramuscular temperature on the duration and amplitude of the individual muscle action potential has been dealt with previously (BENTSEN 1945, BUCHTHAL 1949). In the present study we have studied the effect of intramuscular temperature in two different ways: (1) by examining the effect of different levels of temperature on the mean value of duration and amplitude, and (2) by examining the changes in duration and amplitude of the individual action potentials when the intramuscular temperature was changed continuously.

(1) The effect of muscle temperature on the mean values of action potential duration and amplitude for three different muscles is shown in Table 2. At low temperatures, maximum effort resulted in only a trace of contraction, and therefore only few different potentials could be recorded. The mean duration increased with decreasing temperature, the temperature coefficient being higher, the lower the temperature. Mean duration expressed in per cent of the value at 37° C. varied at 30° C. by approximately 10 per cent per degree, and at 20° C. by approximately 30 per cent per degree. The temperature coefficient varied approximately linearly with the temperature.

The mean amplitude of the muscle action potentials decreased with decreasing intramuscular temperature (Table 2) by about two to five per cent per degree referred to the value at 37° C.

The figures given above apply to simple action potentials only, and polyphasic potentials were not included. They behaved differently both with respect to their occurrence and to their temperature dependence. The percentage of polyphasic potentials increased considerably with decreasing intramuscular temperature. At 37° C., 3 per cent, and in the range down to 30° C., 10—15 per cent of all potentials recorded were polyphasic, while in the range 29—25° C., 30—45 per cent were polyphasic (Table 2). The temperature coefficient of the mean duration of polyphasic action potentials was higher than that of the simple potentials. Thus, at 30° C. their mean duration varied 20 per cent per degree, *i. e.* twice as much as for simple potentials.

(2) The effect of a *continuous* decrease and increase of the intramuscular temperature on the individual action potentials was examined in numerous experiments. However, in only a few was it

Table 2.

Mean values of action potential duration and amplitude at different intramuscular temperatures.

Temperature range (°C)	Degree of activity[1]	Total number of potentials	Number of polyphasic potentials	Simple potentials		Polyphasic potentials	
				Mean duration (msec.)	Mean amplitude (μV)	Mean duration (msec.)	Mean amplitude (μV)
m. abductor digiti quinti							
37—34	5	32	4	7.2	96	9.4	145
34—29	4—5	10	0	9.1	95	—	—
29—25	3	48	22	10.5	80	24.3	160
25—21	2—3	92	40	15.6	70	23.8	190
21—19	2	10	10	—	—	34.1	160
19—14	1	9	5	28.7	55	37.0	170
m. biceps brachii							
37—30	5	140	15	6.8	135	8.7	130
30—25	3—4	110	32	11.3	100	21.1	145
m. extensor digitorum							
37—30	5	85	13	5.9	210	8.4	260
30—25	3—4	70	24	10.2	145	18.1	230

[1] 5 = normal power
4 = active movement against gravity and resistance
3 = active movement against gravity
2 = active movement with gravity eliminated
1 = flicker or trace of contraction (M. R. C. 1943)

possible to follow with certainty the same potential long enough to study the effect both of decreasing and of increasing temperature. Fig. 2 illustrates two successful experiments. The temperature coefficient of action potential duration depended on the duration at 37° C. Thus, the diphasic potential which had a relatively long initial duration (37° C.) varied less with temperature than the short tetraphasic potential (Table 3). There was no hysteresis in duration during the cycle.

The amplitude of the action potential decreased with decreasing temperature. The potential of long duration had a decrease in amplitude of 1 per cent per degree, whereas with the short potential the decrease was 4 per cent per degree (Fig. 2). The hysteresis found for potential amplitude during the cycle of decreasing and increasing temperature was probably caused by small and unavoidable displacements of the recording electrode relative to the active fibres investigated.

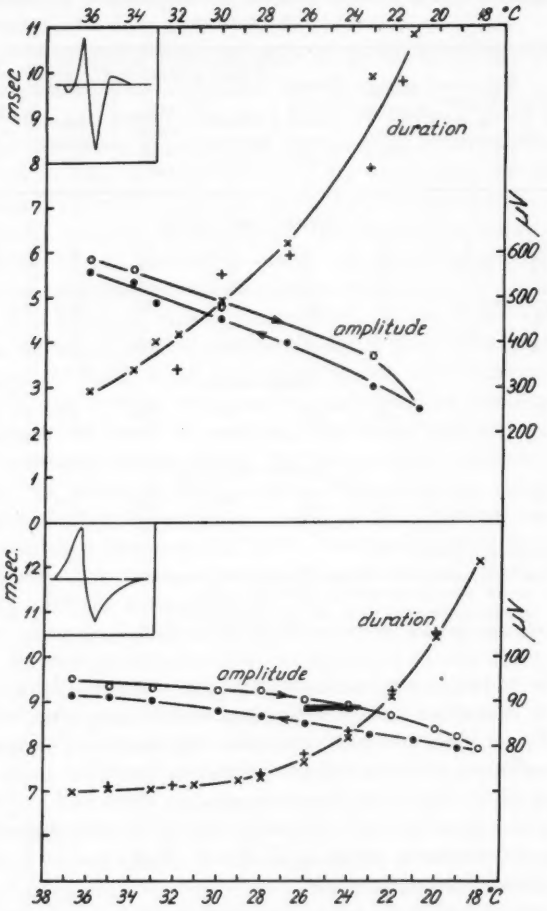


Fig. 2. Duration (left ordinate) and amplitude (right ordinate) as a function of intramuscular temperature for a potential of short duration (upper curves) and of long duration (lower curves) in the m. biceps brachii.

decreasing temperature ○——○ ×——×
increasing temperature ●——● +——+

Table 3.

Temperature coefficient of action potential duration.

(M. biceps brachii.)

Initial duration (msec.)	Temperature range °C	Change in duration per °C in per cent of initial duration
7.0 (diphasic)	37—27	1
	27—18	9
2.9 (tetraphasic)	37—27	6
	27—18	19

4. The action potential parameters as a function of the strength of contraction.

The observations described hitherto referred to action potentials recorded during weak effort. For the synergy of the flexors of the upper arm this amounts to less than the force which compensates the weight of the forearm.

In three different subjects and on different muscles we have determined both amplitude and duration of successively recruited action potentials (Table 4).

Table 4.

Recruitment in normal muscle.

(Bipolar electrodes.)

Muscle	First unit (original)			Second unit (recruited)		
	number of potentials	mean amplitude (μV)	mean duration (msec)	number of potentials	mean amplitude (μV)	mean duration (msec.)
biceps brachii	81	105	4.99	81	208	4.99
abductor digiti quinti	59	56	4.29	59	183	4.05
extensor digitorum communis	27	245	4.16	27	377	4.63

The amplitude of the secondly recruited potential exceeded that of the firstly appearing potential. Thus, in the abductor digiti quinti the mean amplitude of the second potential was 200 per cent higher than that of the first, in the brachial biceps the difference was 100 per cent, and in the extensor digitorum communis it was 50 per cent. There was no difference in mean duration between the original and the recruited action potential.

5. The action potential parameters of fatigued muscle.

Fatigue was produced in the brachial biceps by two minutes of 1 per second maximum contractions. Records were taken immediately after the strong effort by leading off from as many different regions as possible during slight voluntary contraction.

There was no statistically significant difference between the mean duration of fatigued muscle and that of non-fatigued muscle (Table 5). The increase in amplitude in fatigued muscle cannot be considered significant since only two of the five electrodes applied gave a significant increase.

Table 5.

Fatigued and non-fatigued muscle.

(M. biceps brachii.)

State of muscle	Number of potentials	Mean duration (msec.)	Mean amplitude (μV)
fatigued	127	8.37 ± 0.25	270 ± 16
non-fatigued	1,568	7.93 ± 0.07	220 ± 4

However, the percentage of polyphasic action potentials was considerably increased by fatigue. Thus, in some experiments we found an increase of from 8 to 23 per cent.

Discussion.

In the present study a continuous increase in mean action potential duration was found over the range one to eighty years. This is in agreement with PETERSÉN and KUGELBERG (1949) who compared mean duration in subjects 20 to 42 years of age with duration in subjects 69 to 79 years old. If one considers a smaller age range, such as 20 to 40 years, then the change in duration does not appear clearly because of individual variation both in our study and in that of PETERSÉN and KUGELBERG (1949).

The increase in action potential duration might be explained in terms of a decrease in propagation velocity of the impulse over the muscle fibre. Such a change in propagation velocity with age has been demonstrated in motor fibres of the ulnar nerve (WAGMAN and LESSE 1952). The finding is, however, at variance with the change in muscle fibre diameter with increasing age. The mean diameter increases by a factor five up to the age of thirty and then

levels off. One would expect a corresponding increase followed by a decrease in propagation velocity, since there also in muscle is a close relationship between fibre diameter and propagation velocity (HÅKANSSON 1954). This means that the diameter-velocity relationship holds only for muscle fibres under approximately equal physiological conditions.

The increase in mean action potential duration with decreasing intramuscular temperature can similarly be accounted for on the basis of the temperature coefficient of the propagation velocity. The increased occurrence of polyphasic potentials with decreasing temperature can be attributed to a desynchronization of the otherwise simultaneously activated fibres of a motor unit. The decreased propagation velocity of the impulse over the muscle fibre and the terminal nerve fibres may cause a temporal dispersion of fibres within the motor unit (PINELLI and BUCHTHAL 1953). The temporal dispersion would cause a decrease in the mutual coupling of adjacent fibres (KATZ and SCHMITT 1940) and might account for the higher temperature coefficient of polyphasic as compared with simple action potentials.

The increased incidence of polyphasic potentials during fatigue is probably due to an incomplete synchronization between fibres of different motor units, since there was an increased tendency to synchronization between discharges from different motor units in fatigued muscle (BUCHTHAL and MADSEN 1951).

Mean action potential duration did not change with recruitment. In agreement with others, however, the amplitude of the secondly and thirdly recruited units was found to be larger than that of the first. SMITH (1934) explained this increase in amplitude by the decreasing mean distance between the recording electrode and the active unit with increasing strength of contraction. This is consistent with our finding that the distance between the active fibres and the recording electrode is by far the most important determinant of action potential amplitude, more important than the number of activated fibres (BUCHTHAL, GULD and ROSENFALCK 1954 b). Another explanation was offered by DENNY-BROWN and PENNYBACKER (1938) who considered the increase in amplitude to be an expression of the different sizes of the successively activated motor units. KUGELBERG and SKOGLUND (1946) found in fact that electrical stimulation of large diameter nerve fibres evoked muscle action potentials of low amplitude. Assuming a relationship between the height of the muscle spike and

the size of the motor unit they concluded that small motor units are innervated by nerve fibres of large diameter. ECCLES and SHERRINGTON (1930) and CLARK (1931), on the other hand, found that large nerve fibres innervate large motor units, whereas FERNAND and YOUNG (1951) did not find any histological evidence for a correlation between the size of the nerve fibre and the size of the motor unit it innervates. Hence, though it is entirely possible that there is a functional organisation in the sequence of innervation, evidence for its occurrence is still lacking.

Summary.

The influence has been studied of some physiological variables on the duration, amplitude and shape of muscle action potentials in normal subjects. Action potentials were recorded at random with concentric and bipolar needle electrodes during slight voluntary effort.

Mean action potential duration increased continuously with age: from 5.7 msec. at 1 to 4 years to 10 msec. at 80 years of age. The mean amplitude was 192 $\pm$ 2 μV and was independent of age.

With decreasing intramuscular temperature the mean duration increased by 10 to 30 per cent per degree C.; the mean amplitude decreased by 2 to 5 per cent per degree C. The number of polyphasic potentials increased as much as tenfold with a 10 degree decrease in temperature.

Likewise, the incidence of polyphasic potentials increased with fatigue, which did not, however, affect mean action potential duration or amplitude.

Successively recruited action potentials had the same mean duration, while the mean amplitude of the secondly recruited potentials exceeded that of the first by 50 to 200 per cent.

The work was supported by grants from the Michaelsen Foundation and the Rockefeller Foundation.

References.

BENTSEN, K. G., Nord. Med. 1945. *25*. 697.
BUCHTHAL, F., Amer. J. Med. 1949. *6*. 579.
— and A. MADSEN, EEG Clin. Neurophysiol. 1950. *2*. 425.

BUCHTHAL, F. and P. PINELLI, Acta Physiol. Scand. 1951. *25*. Suppl. 89.
— — Acta Med. Scand. 1952. *142*. Suppl. 266. p. 315.
— C. GULD and P. ROSENFALCK, Acta Physiol. Scand. 1954 a. *32*. 200.
— — — Ibidem 1954 b. *31*. Suppl. 114 p. 7.
— P. HÖNCKE and J. LINDHARD, Ibidem 1944. *8*. 230.
CLARK, D. A., Amer. J. Physiol. 1931. *96*. 296.
DENNY-BROWN, D. E. and J. PENNYBACKER, Brain 1938. *61*. 311.
ECCLES, J. C. and C. S. SHERRINGTON, Proc. Roy. Soc. 1930. *B 106*. 326.
FERNAND, V. S. V. and J. Z. YOUNG, Ibidem 1951. *B 139*. 38.
HÅKANSSON, C. H., Acta Physiol. Scand. 1954. *31*. Suppl. 114 p. 22.
KATZ, B. and O. H. SCHMITT, J. Physiol. 1940. *97*. 471.
KUGELBERG, E. and C. R. SKOGLUND, J. Neurophysiol. 1946. *9*. 399.
Medical Research Council. War Memorandum No. 7. Aids to the Investigation of Peripheral Nerve Injuries. H. M. Stationery Office, London 1943.
PETERSÉN, J. and E. KUGELBERG, J. Neurol. Neurosurg. Psychiat. 1949. *12*. 124.
PINELLI, P. and F. BUCHTHAL, EEG Clin. Neurophysiol. 1953. *5*. 589.
SMITH, O. C., Amer. J. Physiol. 1934. *108*. 629.
WAGMAN, J. H. and H. LESSE, J. Neurophysiol. 1952. *15*. 235.

From the Institute of Physiology, University of Lund, Sweden.

Potentiation of Histamine Effects by an Antihistaminase.

By

S.-E. LINDELL and H. WESTLING.

Received 12 May 1954.

When studying the inhibitory action of amidine derivatives on the histaminase of pig kidney, BLASCHKO et al. (1944 and 1951) found that stilbamidine in a concentration of 10^{-3} M completely inhibited the enzyme. In a 10^{-5} M concentration the inhibition was about 50 %.

MONGAR and SCHILD (1951) demonstrated that the histaminase inhibitor semicarbazide selectively potentiated the effect of histamine on an isolated piece of guinea pig's ileum. SMITH (1953) found that stilbamidine increased and prolonged the histamine-induced constriction of isolated swine carotid arteries.

The purpose of the present investigation was to study if histaminase inhibitors modify the response to histamine of a living animal. Guinea pigs were used, since the distribution of histaminase in this animal has been subject to previous investigation (LINDELL and WESTLING 1953). As inhibitor, stilbamidine was chosen. This substance seems to be relatively non-toxic, since it has been used therapeutically in man and experimental animals (SCHOENBACH and GREENSPAN 1948). Since these experiments were completed, ARUNLAKSHANA, MONGAR and SCHILD (1954) have published an extensive study of the potentiation by histaminase inhibitors of the effects of histamine, using mainly isolated organs from the guinea pig. The effect of stilbamidine was not studied.

Experimental.

Histaminase activity in tissue extracts was determined by the method of WICKSELL (1949 a) as used by LINDELL and WESTLING (1953). The effect of stilbamidine in concentrations between 10^{-4} and 10^{-9}M was studied. The amidine was added to the extracts before incubation with histamine.

Female non-pregnant guinea pigs, weighing 500—1,000 g, were anaesthetized with urethane (2 g per kg body weight), half the dose intraperitoneally and the other half subcutaneously. Histamine, 1—20 μg, and stilbamidine, 1—20 mg, were given intravenously through a plastic tube in the jugular or axillary vein. The pressure in the urinary bladder was measured with a water manometer connected to the bladder by a plastic catheter introduced through the urethra. The bronchiolar tone was recorded by the method described by KONZETT and RÖSSLER (1941). When the experiments were completed the animals were killed by bleeding and the lungs, liver kidneys and bladder were removed and extracted for histaminase.

Figures for histaminase activity are given as μg histamine base inactivated in 1 hour by 1 g of the tissue. The figures for histamine refer to the base, whereas those for stilbamidine refer to the 4: 4'-diamidinostilbene-di-(β-hydroxyethanesulphonate), mol. weight 516.6.

Results.

1. Histaminase Activity in Tissue Extracts after Adding Stilbamidine in Vitro.

It was found that stilbamidine diminished the activity of histaminase extracted from lungs, liver and kidneys. A closer analysis of the relation between inhibition and concentration was made with liver extracts from normal animals. Fig. 1 shows that a concentration of 10^{-3} M almost completely inhibited the enzyme. The activity was about half of the normal in a 10^{-7} M solution of stilbamidine and even at 10^{-8} M there was a noticeable inhibition. In concentrations between 10^{-8} M and 10^{-6} M there seemed to be a linear relation between the per cent inhibition and the log of the concentration of stilbamidine. The initial concentration of histamine in these incubations was $10^{-5.7}$ M.

2. Histamine Sensitivity of the Bronchi and Urinary Bladder after Intravenous Injection of Stilbamidine.

The doses of histamine necessary to produce a distinct increase in the bronchiolar tone and pressure in the urinary bladder were

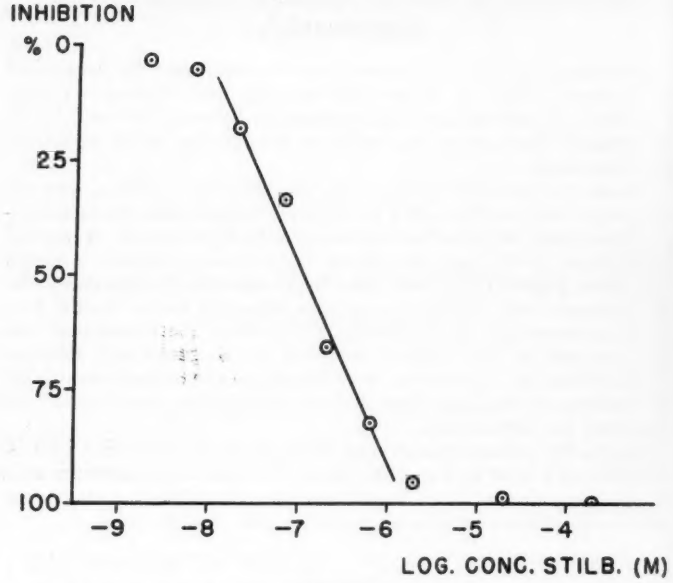


Fig. 1. Mean per cent inhibition of histaminase in guinea pig's liver by various concentrations of stilbamidine. Each point represents the mean of seven observations.

under these conditions, usually between 5 and 10 μg intravenously. These doses caused a contraction of the bronchiolar musculature, which lasted 1—2 minutes. After slow infusion of stilbamidine in doses which themselves had no measurable effect on the bronchi, the response to histamine was distinctly changed. It was usually increased in height, but the most conspicuous change was a prolonged duration. In one typical experiment (Fig. 2) 5 μg of histamine before stilbamidine infusion caused a scarcely noticeable bronchoconstriction, which lasted less than one minute. After 4 mg stilbamidine, the response to 5 μg was increased in height, roughly corresponding to 10 μg of histamine before the stilbamidine infusion. The duration of the response was, however, greatly prolonged, being more than 7 minutes. It is evident that the increased duration is not only due to the increased height, since the response to 10 μg of histamine before stilbamidine lasted only about two minutes. The response to 30 μg of histamine before stilbamidine infusion lasts 4 minutes although it is higher than the response to

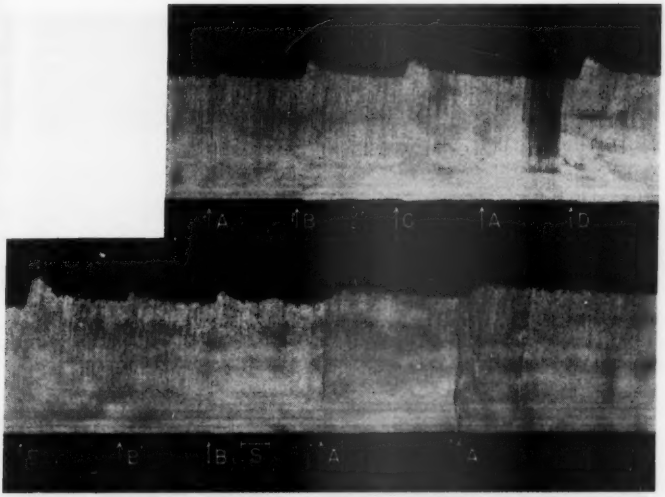


Fig. 2. Bronchiolar tone in female guinea pig, weighing 1,000 g. Time interval: 30 sec. Injections into the jugular vein at A 5 μg, B 10 μg, C 15 μg, D 20 μg and E 30 μg of histamine. At S 4 mg of stilbamidine.

5 μg after stilbamidine. Since the character of the responses to histamine was different before and after stilbamidine infusion no further quantitative comparisons were made.

In some experiments the pressure in the urinary bladder was registered. As shown by WICKSELL (1949 b), histamine causes a contraction of the smooth musculature in the bladder resulting in an increase in pressure. Although the interpretation of the records sometimes presented difficulties owing to spontaneous

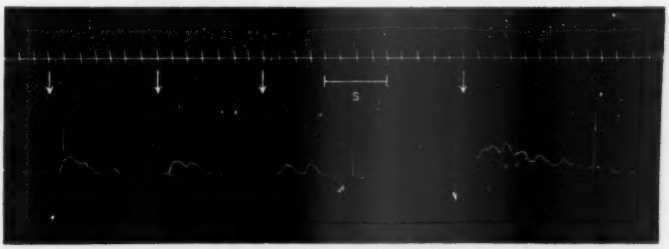


Fig. 3. Urinary bladder pressure in female guinea pig, weighing 800 g. Time interval: 30 sec. Arrows indicate injections of 20 μg histamine in the jugular vein. At S 10 mg stilbamidine per kg were infused.

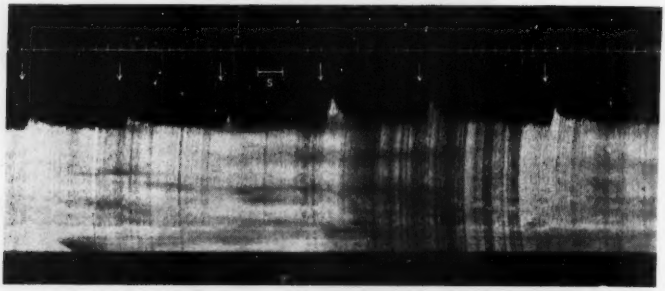


Fig. 4 a. Bronchiolar tone in female guinea pig, weighing 850 g. Time interval: 30 sec. Arrows indicate injections of 10 μg histamine into the jugular vein. At S 4 mg stilbamidine per kg were infused.

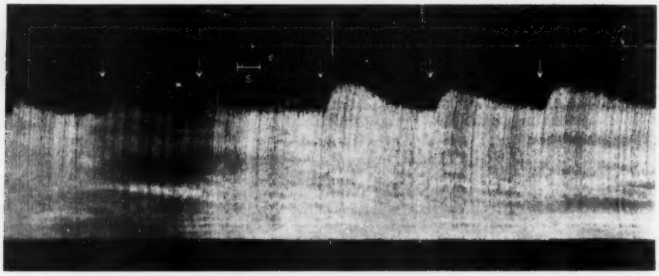


Fig. 4 b. Same experiment as in Fig. 5. Arrows: injections of 10 μg histamine. At S 8 mg stilbamidine per kg were infused.

contractions it was usually possible to demonstrate an increase of the responses to histamine after stilbamidine infusion (Fig. 3), the most conspicuous change being an increased duration.

The minimal potentiating dose of stilbamidine varied with different guinea pigs. Yet, in one and the same animal, potentiation was greater with increasing doses of stilbamidine. Fig. 4 a and b show records of histamine-induced bronchoconstriction in the same animal. Compare the potentiation obtained by 4 and 8 mg of stilbamidine. After small doses of stilbamidine there was a gradual return to normal sensitivity (Fig. 4 a).

It is known that the sensitivity of guinea pigs to histamine varies with the depth of anaesthesia, being higher at more superficial levels. To exclude this source of error additional doses of urethane were given before the infusion of stilbamidine.

3. Histaminase Activity in Various Organs after Intravenous Injection of Stilbamidine.

Table 1 shows the histaminase activity in the lungs, liver, kidneys and urinary bladder removed from animals used in the previous experiments. The animals are divided into 4 groups, one comprising animals which had not received stilbamidine and the others, animals injected with stilbamidine in increasing doses.

Table 1.

Mean values for histaminase activity in organs from the guinea pigs used. Figures within brackets denote number of animals.

Tissue	Histaminase activity µg/g/hr			
	No stilbamidine	Dose of stilbamidine mg/kg body weight		
		1—5	5—10	10—16.5
Liver	15.1 (10)	10.3 (6)	6.7 (6)	3.3 (5)
Lung	0.4 (10)	0.3 (4)	0.2 (5)	0.1 (5)
Kidney	2.0 (10)	1.1 (6)	0.6 (6)	0.3 (5)
Bladder	6.8 (10)	5.1 (4)	4.1 (5)	3.5 (5)

Stilbamidine in doses of 1—5 mg/kg body weight reduced the histaminase activities in the organs studied by 25 % on the average. 5—10 mg and 10—16.5 mg/kg of stilbamidine resulted in average reductions of 50 and 75 % respectively. The bladder seems to differ somewhat from the other organs studied, its histaminase activity not being diminished to the same extent.

Discussion.

Confirming BLASCHKO et al. the present experiments show that stilbamidine is a potent inhibitor of histaminase. Whereas these investigators found a 10^{-3} M concentration to be necessary for complete inhibition we observed that stilbamidine in a 10^{-5} M solution practically abolished the activity of the enzyme. A similar discrepancy holds for 50 % inhibition. There are, however, differences in experimental conditions: BLASCHKO et al. worked with an enzyme preparation from an acetone-dried powder of pig kidney using a manometric method measuring oxygen consumption.

The substrate was cadaverine. We have used a crude extract from the homogenized guinea pig liver and a different method of assessing histaminase activity.

When stilbamidine was given by vein to a guinea pig, organs normally rich in histaminase showed diminished enzyme activity on extraction. The doses required for inhibition were larger than would be expected from the experiments in vitro. The animals were, however, killed 15 to 60 minutes after the infusion of stilbamidine and it is known that intravenously injected stilbamidine rapidly disappears from the blood (SCHOENBACH and GREENSPAN 1948), the mode of elimination of the drug being unknown. It is possible that stilbamidine is inactivated in the tissues, a view that is supported by the observation that the potentiation of histamine effects is reversible.

It seems likely that the potentiation of histamine effects by stilbamidine is due rather to diminished inactivation of histamine than to rendering the specific effector cells more sensitive, since the potentiation is mainly characterized by a prolongation of the response. Histamine injected intravenously to cats is rapidly removed from the blood, predominantly in organs that are rich in histaminase (EMMELIN 1951) and as shown above stilbamidine inhibits the histaminase in the liver and kidneys, which are the main depôts of histaminase in the guinea pig (LINDELL and WESTLING 1953). The potentiating effect of stilbamidine might, however, also be due to the diminished histaminase activity in the effector organs, lungs and bladder, that was demonstrated above. The histaminase activity in the non-pregnant female guinea pig's plasma (AHLMARK 1944) is too low to be of any importance in these experiments.

The comprehensive studies by SCHILD and his group strongly indicate a correlation between antihistaminase and histamine potentiating properties (ARUNLAKSHANA, MONGAR and SCHILD 1954).

MAC INTOSH and PATON (1949) found that stilbamidine given intravenously to cats raised the level of histamine in blood and we observed that stilbamidine, injected rapidly, sometimes caused a bronchoconstriction. Some of the side effects of stilbamidine, when used therapeutically i. a. facial flushing, dyspnoea, fall in blood pressure and oedema may be caused by an increase in blood histamine, an increase that may, at least in part, be due to inhibition of histaminase and concomitant accumulation of histamine.

It seems probable that substances which inhibit histaminase in the living animal might be useful in studies on the physiological importance of histaminase. They may also become valuable tools for studies of the rôle of histamine in various physiological and pathological conditions.

Summary.

1. In guinea pigs the histaminase activity in lungs, liver, kidney and urinary bladder was reduced after intravenous injections o stilbamidine.

2. Doses of stilbamidine, effective in inhibiting histaminase, potentiated the bronchoconstriction and increase in urinary bladder tone caused by histamine given intravenously.

This work was aided by a grant from the Medical Faculty, University of Lund. Technical assistance was given by Miss Edith Lehmann.

References.

Ahlmark, A., Acta Physiol. Scand. 1944. *9.* suppl. 28.
Arunlakshana, O., J. L. Mongar and H. O. Schild, J. Physiol. 1954. *123.* 32.
Blaschko, H. and R. Duthie, Biochem. J. 1944. *38.* XXV.
— H., F. N. Fastier and Isabelle Wajda, Ibid. 1951. *49.* 250.
Emmelin, N., Acta Physiol. Scand. 1951. *22.* 379.
Konzett, H. and R. Rössler, Arch. exp. Path. Pharmak. 1940. *195.* 71.
Lindell, S.-E. and H. Westling, Acta Physiol. Scand. 1953. *30.* suppl. 111, p. 202.
Mac Intosh, F. C. and W. D. M. Paton, J. Physiol. 1949. *109.* 190.
Mongar, J. L. and H. O. Schild, Nature 1951. *167.* 232.
Schoenbach, E. B. and E. M. Greenspan, Medicine 1948. *27.* 327.
Wicksell, F., Acta Physiol. Scand. 1949. *17.* 359.
— Ibid. 1949. *17.* 415.

From the Physiological Institute, University of Uppsala.

The Rôle Played by the Pyloric Region in the Cephalic Phase of Gastric Secretion.

By

SVEN LINDE.

Received 17 May 1954.

The existence of a hormonal mechanism for the gastric phase of gastric secretion is now accepted by most investigators.

As to the cephalic phase of gastric secretion UVNÄS (1942) suggested that stimulation of the vagus nerves to the stomach results in the release of gastrin and that neither the vagal nor the gastrin mechanism can operate to its full extent in the absence of the other. EMMELIN and KAHLSON (1944) have extended this theory and suggest, that during the cephalic phase there exists a "two-stage humoral mechanism", the first stage involving the liberation of gastrin, histamine being the second and final link.

UVNÄS's theory has been criticised by some authors. BABKIN and SCHACHTER (1944) repeated UVNÄS's experiments on dogs and confirmed his findings, that the secretion during vagal stimulation was inhibited by pylorectomy. The inhibition was however transient, and they assumed that it was due to hyperactivity of the sympatho-adrenal system, depending on the crude handling of the stomach during the operation. GROSSMAN (1950) is doubtful about UVNÄS's theory, because of the fact that insulin hypoglycemia in an Heidenhain pouch dog produces strong vagal stimulation of the main stomach with high acid production, yet no secretory response occurs in the pouch. If gastrin is released, it ought to circulate in the bloodstream and cause stimulation of the pouch.

In an earlier investigation by the present author (LINDE 1950), these problems were examined. The results showed that during the cephalic phase of gastric secretion the pyloric region is of the greatest importance to the secretion of acid and water. It was

further shown that it is possible to inhibit the secretion during vagal stimulation by specific antihistaminic substances when a detergent — Tween 20 — was administered simultaneously. These findings support the theory of EMMELIN and KAHLSON that two humoral factors — gastrin and histamine — co-operate during the cephalic phase.

However other results indicated that the pyloric region is of very little or no importance to the activity of the pepsin-producing cells. These cells may possibly be stimulated directly by acetylcholine liberated by the vagus nerve.

The results of experiments described in the present paper are in agreement with those described above. The experiments were performed in two different ways. In the first place, the latency time of the acid and pepsin secretion during vagal stimulation was determined. Secondly, experiments were performed where acetylcholine was instilled into the stomach. All experiments were done on cats.

A. The Latency Time of the Gastric Secretion During Vagal Stimulation.

Several authors have observed that the latent period of the secretion induced by vagal stimulation is exceptionally long. VINEBERG (1931) found an average latent period of twenty-seven minutes in sixteen experiments on dogs.

It might be possible that this fairly long latent period is due to the development time of the "hormonal chain" acetylcholin—gastrin—histamine. According to the results referred to above, the hormonal mechanism is of very little or no importance to the secretion of pepsin however. The latent period of the secretion of pepsin ought therefore to be much shorter than that of the acid secretion.

Experimental Procedure.

9 cats were anaesthetized with chloralose-urethane. The pylorus and cardia were ligated. The cats were placed in the supine position and a Pezzer catheter was introduced at the deepest point of the stomach in this position, *i. e.* the back wall of the fornix, and pushed out through an incision in the back. The gastric juice was collected in a special measuring cylinder attached to the rubber catheter.

To obtain a constant basal secretion, histamine was administered by

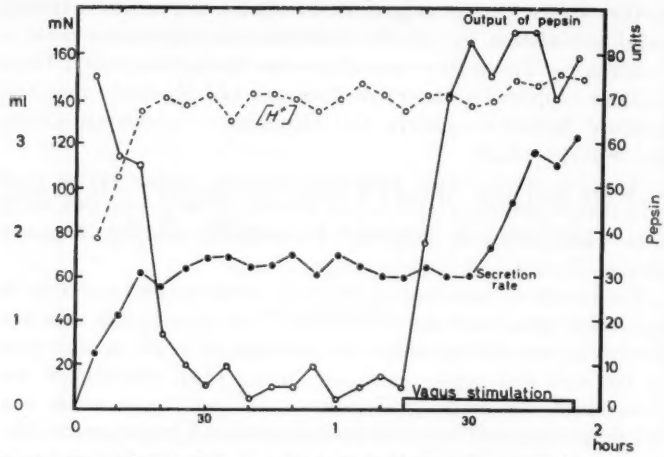


Fig. 1. The different latent periods of the increased output of pepsin and secretion rate after vagus stimulation.

continuous intravenous injection (0.4—0.5 mg/hour). The vagi were cut in the neck and introduced into a glass tube between silver electrodes. The electric stimulus was derived from the repeated discharge of a condensor.

The measuring cylinder was emptied every 5 minutes. The volume and the total acidity of the gastric juice secreted in 5 minutes was determined. The concentration of pepsin was estimated by Hunt's method (1948).

After having obtained a constant secretory rate for at least one hour by giving histamine, strong vagal stimulation was induced. The latent period between the starting of the vagal stimulation and the observed increase of the secretory rate varied between 15 and 25 minutes in these 9 experiments. The peptic activity decreased in all experiments to very low values before the stimulation of the vagi. Even in the first sample of gastric juice obtained 5 minutes after the beginning of the vagal stimulation, the concentration of pepsin had increased to fairly high values in all cases. One of the experiments is illustrated in Fig. 1.

B. Instillation of Acetylcholine into the Stomach.

A comparatively large subcutaneous dose of acetylcholine has to be used in order to produce gastric secretion in a cat. The

reason for this is undoubtedly the destruction of acetylcholine by esterase.

ROBERTSON et al. (1950) showed however, that irrigation of a pyloric pouch with acetylcholine produced acid secretion by the fundic glands in dogs. The peptic activity of the gastric juice secreted was low. Instillation of acetylcholine into the fundic part of the stomach produced no stimulation of acid secretion.

In the opinion of the present author (cf. LINDE 1950), the pepsin producing cells are stimulated directly by acetylcholine released by the vagal stimulation, while the parietal cells are stimulated by a hormonal mechanism involving acetylcholine—gastrin—histamine. The results described below are in agreement with this hypothesis.

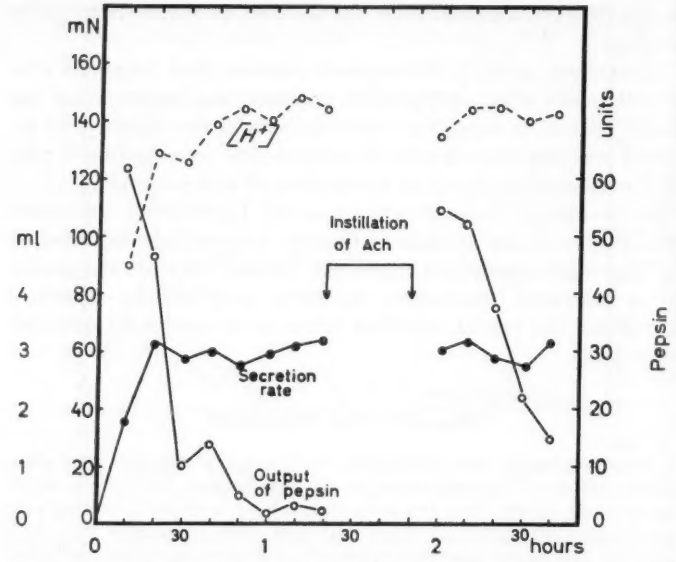
Experimental Procedure.

The experiments were performed on 6 cats anaesthetized with chloralose-urethane. The pylorus and cardia were ligated. The pyloric region was then separated from the main stomach by a suture involving only the mucous membrane. Rubber catheters were introduced into both pouches. The gastric juice was collected in a measuring cylinder attached to a Pezzer catheter introduced into the main stomach. The cylinder was emptied every 10 minutes. The secretory rate, the acidity, and the peptic activity were determined for every sample.

In each case 20 ml of 2 % acetylcholine chloride was first introduced into the pyloric part of the stomach with low infusion pressure (2—5 cm). The pyloric pouch was emptied every 30 minutes and fresh acetylcholine solution introduced. After a latent period of 10—30 minutes a secretion of gastric juice from the main stomach was obtained in all cases. The maximal secretory rate varied between 0.8 and 2.4 ml/10 minutes and the maximal total acidity between 62 and 138 mN. In all experiments the peptic activity as well as the output of pepsin decreased to very low values during the first hour.

In 4 of the cats 20 ml of physiological saline solution was instilled into the pyloric pouch before the introduction of acetylcholine. No stimulation of acid secretion occurred.

After the preliminary experiments the pyloric pouch was washed with physiological saline solution. Histamine was then administered continuously intravenously (0.4—0.5 mg/hour). When the secretory rate after about one hour was fairly constant, 30 ml of 2 % acetylcholine chloride was introduced into the main stomach at low pressure and left there for 30 minutes. In all cases the peptic activity decreased to very low values during the period before the instillation of acetylcholine. In the first samples obtained after



The increased output of pepsin after instillation of acetylcholine in a fundic pouch.

the instillation the peptic activity was very high, and then decreased again. In no case was an increase of the secretory rate or the acidity observed. A typical experiment is demonstrated in Fig. 2.

Discussion.

The results of this investigation support the idea that the secretion of acid and pepsin during vagal stimulation are initiated by different mechanisms, the acid secretion being induced by a neurohormonal mechanism involving the chain acetylcholine—gastrin —histamine, and the pepsin secretion directly by acetylcholine (cf. LINDE 1950). The results also indicate that acetylcholine is able to release gastrin from the pyloric mucosa and this is able to stimulate the parietal cells of the fundic mucosa. The secretion of pepsin, on the other hand, is induced only when acetylcholine comes in direct contact with the pepsin producing cells.

This gives support to the theory of UVNÄS that vagal stimulation is able to release gastrin. If local irrigation of the pyloric

mucosa by acetylcholine is able to stimulate acid secretion from the fundus, while irrigation of the fundic mucosa has no such effect, it seems most probable that acetylcholine liberated by vagal stimulation is able to release gastrin too. It is, however, difficult to explain why a vagally denervated pouch does not secrete during vagal stimulation, while a fundic pouch with the vagi cut is able to secrete by instillation of acetylcholine into the pyloric part (cf. GROSSMAN et al. 1950). It is possible that the amount of gastrin released by vagal stimulation is too small to cause secretion from a denervated fundic pouch, but large enough to stimulate the fundic glands when they are "sensitized" by acetylcholine liberated from the vagi. After vagotomy there is a decrease in the responsiveness of the parietal cells to injection of gastrin (ANTIA and IVY 1949).

As seen from the results above, the parietal cells are not stimulated by acetylcholine in direct contact with them in contrast to the pepsin producing cells, which are stimulated directly. The difference in the latency time shows that the two sets of cells are stimulated in different ways during vagal stimulation. In an earlier investigation (LINDE 1950), it was shown that a humoral mechanism is of very little or no importance to the secretory activity of the pepsin producing cells. These cells may possibly be stimulated direct by acetylcholine liberated by the vagus nerves. The results of the present investigation confirm this assumption. Irrigation of the pyloric mucosa by acetylcholine had no stimulatory effect on the secretion of pepsin from the fundus, while irrigation of the fundic mucosa caused a strong stimulation.

Summary.

In cats with whole stomach pouches a constant secretory rate was obtained by continuous intravenous injection of histamine. The output of pepsin decreased to very low values. Without stopping the injection of histamine, the vagi in the neck were then stimulated electrically. The secretory rate and the acidity increased after a latent period varying between 15 and 25 minutes. The output of pepsin on the other hand increased to fairly high values even during the first 5 minutes after the beginning of the vagal stimulation.

In other series of experiments on cats, the stomach was di-

vided into a fundic and a pyloric pouch by a suture through the mucosa only. Instillation of acetylcholine into the pyloric pouch caused an acid secretion from the fundic pouch. The output of pepsin decreased to very low values during this part of the experiments.

In the same cats a constant secretory rate from the fundic pouch was then obtained by continuous intravenous injection of histamine. After about one hour the output of pepsin was very low. Without stopping the injection of histamine, acetylcholine was then instilled into the fundic pouch and left there for half an hour. After the instillation, the peptic activity increased to very high values while the secretory rate and the acidity remained unchanged.

The results of this investigation give support to the theory that the acid and the pepsin secretion during vagal stimulation are initiated by different mechanisms. The acid secretion is assumed to be stimulated by a neuro-hormonal mechanism involving the chain acetylcholine—gastrin—histamine, while the secretion of pepsin is stimulated directly by acetylcholine liberated by the vagus nerves.

References.

ANTIA, F. and A. C. IVY, Fed. Proc. 1949. *8*. 5.
BABKIN, B. P. and M. SCHACHTER, McGill Med. J. 1944. *13* 127.
EMMELIN, N. and G. S. KAHLSON, Acta Physiol. Scand. 1944. *8*. 289.
GROSSMAN, M. I., Physiol. Rev. 1950. *30*. 33.
HUNT, J. N., Biochem. J. 1948. *42*. 104.
LINDE, S., Acta Physiol. Scand. 1950. *21*. Suppl. 74.
ÖBRINK, K. J., Ibidem 1948. *15*. Suppl. 51.
ROBERTSON, C. R., K. LANGLOIS, C. G. MARTIN, G. SLEZAK and M. I. GROSSMAN, Amer. J. Physiol. 1950. *163*. 27.
UVNÄS, B., Acta Physiol. Scand. 1942. *4*. Suppl. 13.
VINEBERG, A. M., Amer. J. Physiol. 1931. *96*. 363.

From the Research Institute of National Defense, Medical Department[1], Sundbyberg, Sweden, and Medicinska Nobelinstitutet, Biokemiska Avdelningen[2], Stockholm, Sweden.

The Antidote Effect of Some Sulfur Compounds and Rhodanese in Experimental Cyanide Poisoning.

By

CARL-JOHAN CLEMEDSON[1], HOLTER I:SON HULTMAN[1] and BO SÖRBO[2].

Received 28 May 1954.

In the treatment of cyanide poisoning, different compounds have been suggested for use as antidotes. Some of these owe their antidote effect to their ability to form complexes with cyanide, others are different sulfur containing compounds. The latter were introduced by S. LANG (1895), who reported that especially sodium thiosulfate was an active antidote against cyanide poisoning. The positive results with thiosulfate were later confirmed by others (TEICHMAN and NAGEL 1919, FORST 1928, CHEN, ROSE and CLOWES 1934, WIRTH 1935). The antidote effect of thiosulfate was apparently explained, when K. LANG (1933) discovered an enzyme, named rhodanese, which catalyzed the reaction between thiosulfate and cyanide, giving the much less toxic compounds thiocyanate and sulfite. Rhodanese, which occurs especially in the liver and kidneys of higher animals, has recently been purified and crystallized (SÖRBO 1953) and the possibility of potentiating the antidote effect of injected thiosulfate by simultaneously injecting the pure enzyme, has now been investigated in the present work. The antidote effect of thiosulfate alone and of two other sulfur compounds, cysteine and tetrathionate, previously reported to be of value as antidotes in cyanide poisoning, has been reinvestigated.

Methods.

The experiments were carried out on 153 adult rabbits of 2—3.5 kg body weight. Cyanide was administered as KCN in saline solution, at the beginning of the investigation neutralized with an equivalent amount of HCl. This neutralization is not necessary, however, as the buffering capacity of the body fluids is sufficient to convert the injected cyanide ion into the free acid. The sulfur compounds were injected intravenously in one of the marginal ear veins immediately prior to the injection of the cyanide. The latter was administered by a rapid intravenous injection in another ear vein (time of injection 30 seconds). When the cyanide was injected subcutaneously, the antidote was administered intravenously as soon as the first toxic symptoms appeared (cf. CHEN et coll. 1934). LD_{50} for cyanide was in each case obtained by gross inspection of the data obtained. Rhodanese activity in homogenates of liver and kidneys from cyanide injected and control animals was determined according to SÖRBO (1953). Crystalline rhodanese from beef liver was prepared according to a previously published procedure (SÖRBO 1953) and remaining ammonium sulfate was removed by dialysis against 0.01 M sodium acetate. Tetrathionate was prepared according to GILMAN et coll. (1946). Other compounds used were of the highest purity commercially available.

Results.

The toxicity of cyanide administered by a rapid intravenous (table I) and subcutaneous (table II) injection was first determined. The LD_{50} was in the former case obtained as about 0.7 mg HCN/kg and in the latter case as about 3 mg HCN/kg. These results agree with those obtained by earlier investigators. The effect of thiosulfate on the toxicity of intravenously and subcutaneously injected cyanide is shown in table III and IV respectively. The LD_{50} was now in the first case about 0.9 mg/kg and in the second case about 4 mg HCN/kg or a little more than without thiosulfate. If, however, rhodanese is injected together with thiosulfate, a significant increase of the LD_{50} is obtained, as shown in table V and VI. The LD_{50} is now about 3.4 mg/kg and about 45 mg/kg respectively, or a 4-fold respectively 10-fold increase over the values obtained without the enzyme. Finally the effect of cysteine and tetrathionate on the toxicity of intravenously injected cyanide is shown in table VII. The effect of cysteine is insignificant, but tetrathionate has a definite antidote

Table I.

Toxicity of intravenously injected cyanide.

Dose mg HCN/kg body weight	Results	
	Surviving	Dead
0.5	3	0
0.6	4	1
0.7	5	4
0.8	3	8
0.9	4	9
1.0	1	6
1.1	0	3
LD_{50} = about 0.7 HCN/kg body weight		

Table II.

Toxicity of subcutaneously injected cyanide.

Dose mg HCN/kg body weight	Results	
	Surviving	Dead
2.5	4	0
3.0	1	2
3.5	1	2
3.8	1	2
4.0	0	2
LD_{50} = about 3 mg HCN/kg body weight		

Table III.

Toxicity of intravenously injected cyanide after a previous injection of thiosulfate.

Thiosulfate dose = 4-fold molar equivalent of cyanide.

Dose mg HCN/kg body weight	Results	
	Surviving	Dead
0.7	7	0
0.8	2	3
0.9	2	1
1.2	0	3
LD_{50} = about 0.9 mg HCN/kg body weight		

Table IV.

Toxicity of subcutaneously injected cyanide after injection of thiosulfate.

Thiosulfate dose = 4-fold molar equivalent of cyanide.

Dose mg HCN/kg body weight	Results	
	Surviving	Dead
3.5	3	0
4.0	3	1
4.5	0	3
LD_{50} = about 4 mg HCN/kg body weight		

Table V.

Toxicity of intravenously injected cyanide after a previous injection of thiosulfate and rhodanese.

Thiosulfate dose = 4-fold molar equivalent of cyanide, rhodanese dose = 750 RU/kg body weight.

Dose mg HCN/kg body weight	Results	
	Surviving	Dead
2.8	3	0
3.1	4	2
3.4	3	1
3.7	0	3
LD_{50} = about 3.4 mg HCN/kg body weight		

Table VI.

Toxicity of subcutaneously injected cyanide after injection of thiosulfate and rhodanese.

Thiosulfate dose = 4-fold molar equivalent of cyanide, rhodanese dose = 750 RU/kg body weight.

Dose mg HCN/kg body weight	Results	
	Surviving	Dead
35	2	0
40	3	0
45	1	3
60	0	3
65	0	2
LD_{50} = about 45 mg HCN/kg body weight		

Table VII.

Toxicity of intravenously injected cyanide after a previous injection of cysteine or tetrathionate.

Cysteine dose = 4-fold molar equivalent of cyanide, tetrathionate dose = 50 mg/kg body weight.

Dose mg HCN/kg body weight	Antidote	Results	
		Surviving	Dead
0.9	Cysteine	3	0
1.1	»	0	3
1.2	Tetra-thionate	3	0
1.5	»	2	1
1.8	»	1	2
2.3	»	1	4
2.8	»	0	1
3.4	»	0	2

LD_{50} = about 1.0 mg HCN/kg cysteine and about 1.7 mg HCN/kg body weight for tetrathionate.

effect. The dose of the latter compound used was the highest one, which does not cause kidney lesions in rabbits (GILMAN et coll. 1946).

Discussion.

The present investigation has shown that thiosulfate alone is without any significant value as an antidote against cyanide, in contrast to earlier reports (LANG 1895, TEICHMAN and NAGEL 1919, FORST 1928, CHEN et coll. 1934, WIRTH 1935). It must be pointed out that these previous results have been obtained with a 100-fold higher dose of antidote on a small number of animals and with techniques, different from those used in the present investigation. HIMWICH and SAUNDERS (1948) have pointed out, that there is a discrepancy between the small antidote effect of thiosulfate reported in the literature, and the considerable activity of rhodanese in the animal body. The yat-tributed this discrepancy to the low permeability of thiosulfate through the cell membranes, which must necessarily restrict the antidote effect of thiosulfate, as rhodanese is present only intracellularly. K. LANG (1949) on the other hand had found that rhodanese was inhibited by cyanide and then suggested that the enzyme was inactivated in cyanide poisoned animals. In the light of other experiments on the cyanide inhibition of rhodanese

Table VIII.

Rhodanese activity in cyanide poisoned and control animals.

Cyanide poisoned rabbits were killed by a rapid intravenous injection of 1.1 mg HCN/kg body weight. Controls were killed by a blow on the head.

Treatment	Liver rhodanese RU/g fresh weight	Kidney rhodanese RU/g fresh weight
Control	147	45
»	176	46
»	180	51
Cyanide	130	62
»	129	45
»	128	52

(SÖRBO 1951) this explanation was considered as very dubious. The following experiments were consequently carried out. Rabbits were injected with a lethal dose of cyanide, and the rhodanese activity in a 10 % homogenate of the liver and kidneys was determined and compared with the values obtained from normal animals (killed by a blow on the head). As shown in table VIII, only an insignificant decrease of the rhodanese activity in the liver was encountered and no decrease at all in the kidneys. Our results thus disprove the theory of LANG (1949). A previous claim by VOEGTLIN, JOHNSON and DYER (1926) that cysteine is an effective antidote in cyanide poisoning has also been disproved in the present work, which could be expected, as cysteine is not a substrate for rhodanese. On the other hand the antidote effect of tetrathionate (DRAIZE 1933, CHEN, ROSE and CLOWES (1933/1934) was confirmed. This compound spontaneously reacts with cyanide (see KURTENACKER 1938) giving thiocyanate, thiosulfate and sulfite. Its therapeutic effect is, however, limited by its nephrotoxic action (GILMAN et coll. 1946).

The question of which antidote should be used in the treatment of cyanide poisoning is difficult to answer definitely. CHEN and ROSE (1952) have reported good therapeutic results in man with a combination of nitrite and thiosulfate, the former compound acting by converting hemoglobin to hemiglobin, in which the trivalent iron combines with cyanide. In the light of the present investigation, the value of thiosulfate seems to be inferior to that of tetrathionate. Concerning the use of rhodanese preparations as an antidote in cyanide poisoning, a detailed study will be published later.

Summary.

1. The LD_{50} for cyanide is about 0.7 mg HCN/kg body weight, when administered intravenously and about 3 mg/kg when injected subcutaneously in rabbits.

2. Thiosulfate, in a 4-fold excess over cyanide, is without any significant effect on these values.

3. A combination of thiosulfate and rhodanese increases the LD_{50} for intravenously injected cyanide to about 3.4 mg/kg and for subcutaneously injected cyanide to about 45 mg/kg.

4. Tetrathionate increases the LD_{50} for intravenously injected cyanide to about 1.7 mg/kg. Cysteine is without any significant effect.

5. Rhodanese activity in liver and kidneys is not significantly impaired in cyanide poisoned animals.

References.

Chen, K. K., C. L. Rose and G. H. A. Clowes, Proc. Soc. Exp. Biol. N. Y. 1933. *31*. 250.
— — — J. Pharmacol. 1934. *51*. 132.
— — J. Amer. Med. Ass. 1952. *149*. 113.
Draize, J. H., Science 1933. *78*. 145.
Forst, A. W., Arch. exp. Path. Pharmak. 1928. *128*. 1.
Gilman, A., F. S. Philips, E. S. Koelle, R. P. Allen and E. St. John, Amer. J. Physiol. 1946. *147*. 115.
Himwich, W. A. and J. P. Saunders, Ibidem 1948. *153*. 348.
Kurtenacker, E., Chemie der Sauerstoffsäuren des Schwefels. Stuttgart 1938.
Lang, S., Arch. exp. Path. Pharmak. 1895. *36*. 75.
Lang, K., Biochem. Z. 1933. *259*. 243.
— Z. Hormon-, Ferment-, Vitaminforsch. 1949. *2*. 288.
Sörbo, B. H., Acta Chem. Scand. 1951. *5*. 724.
— Ibidem 1953. *7*. 1129.
Teichman, E. and W. Nagel, Biochem. Z. 1919. *93*. 312.
Voegtlin, C., J. M. Johnson and H. A. Dyer, J. Pharmacol. 1926. *27*. 467.
Wirth, W., Arch. exp. Path. Pharmak. 1935. *179*. 558.

From the Institute of Neurophysiology, University of Copenhagen and the Department of Ear-, Nose- and Throat-Diseases, University Hospital of Copenhagen.

The Influence of Acetylcholine-like Substances, Menthol and Strychnine on Olfactory Receptors in Man.

By

ARNE P. SKOUBY and K. ZILSTORFF-PEDERSEN.

Received 28 May 1954.

It has previously been demonstrated that cold and pain receptors in the human skin (BING and SKOUBY 1950, SKOUBY 1951, 1953), thermal receptors of the cat's tongue (DODT, SKOUBY and ZOTTERMAN 1953) and baroceptors of the carotid sinus of the cat (LANDGREN, SKOUBY and ZOTTERMAN 1953) are sensitized to their adequate stimuli by small amounts of acetylcholine, while larger amounts exert an inhibiting effect. Acetylcholine in itself does not evoke sensations.

The chemoceptors to smell represented by the ganglion cells of the olfactory mucosa differ from all other sensory organs in that they are at the same time receptor cells and ganglionic cells serving the double function of reception and conduction. The purpose of the present study was to investigate whether acetylcholine and acetylcholine-like substances also have a sensitizing effect when applied to this special type of chemoreceptor.

Menthol influences thermoceptors (HENSEL and ZOTTERMAN 1951, DODT, SKOUBY and ZOTTERMAN 1953), baroceptors and chemoceptors of the carotid area (LANDGREN, SKOUBY and ZOTTERMAN 1953) in a similar way as acetylcholine and its effect on the chemoceptors of smell was studied as well.

Finally the sensitizing effect of strychnine on smell receptors, originally claimed by FRÖHLICH (1851) but denied by FILEHNE (1901), was reinvestigated.

Technique.

The subjects were female and male students, 22—29 years of age. Their sensitivity to olfactory stimuli was measured by the method described by ELSBERG and LEVY (1935) using coffee as the smell stimulus. Air provided from a 500 ml container with 30 ml grained coffee on its bottom was used as the stimulus. The smell threshold was determined as the mean value of the lowest volume of air eliciting and the highest not eliciting smell sensation, the difference between the two volumes being 0.25 ml.

The spontaneous variation in smell threshold was investigated in repeated tests on ten subjects. Four individuals, two females and two males, were selected in whom uniform values for the smell threshold were obtained over 15 minute periods and no decreases exceeding 10 per cent of the initial value occurred in the course of an hour. The thresholds were determined at each nostril, allowing one minute between stimulations. There were frequently differences in threshold between the right and the left olfactory area, of up to 400 per cent (= 4 ml). For the same area the threshold varied from day to day and from individual to individual (table 1).

Table 1.

Smell threshold of right and left sides of two subjects on six different days.

Experimental days.			1.	2.	3.	4.	5.	6.
Subjects.	M.	r.	3.37	3.37	2.63	2.12	2.12	2.37
		l.	2.63	1.87	2.12	0.87	2.37	1.87
	R.	r.	6.12	2.37	5.87	2.12	1.87	5.12
		l.	2.12	4.63	2.12	4.63	4.12	1.12

Due to the hidden localization of the olfactory area, direct and well defined application of the chemical agents to be studied was not possible. The use of atomizers gave inconsistent results. Therefore 0.1 ml of the test solution (2 drops; 37°C) was dropped in the subject's nostril from a pipette. The subject lay on his back, with the head extended so that the chin and the external auditory canals lay in the same vertical plane. The tip of the pipette was placed between the nasal septum and the anterior part of the median concha. The solution to be tested was applied when the smell threshold had been constant for a period of 15 minutes.

Water free acetylcholine and acetyl-beta-methylcholine were dissolved immediately before use in 0.9 per cent NaCl solution to give a concentration of 0.1 μg to 1 mg per ml. Stock solutions of menthol (0.04 per cent) in water, and strychnine nitrate (1 per cent) in 0.9 per cent saline were diluted with 0.9 per cent saline to give the desired concentrations.

For controls 0.9 per cent NaCl was used.

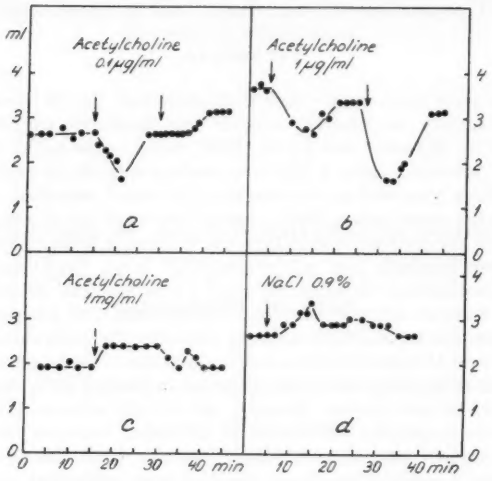


Fig. 1. Smell threshold variations of one olfactory area in subject M. after application of:

a. 0.1 µg acetylcholine per ml.
b. 1 µg » » »
c. 1 mg » » »
d. 0.9 per cent NaCl.
The time of application is marked by the arrow.
Ordinate: smell threshold in ml air.
Abscissa: time in minutes.

Results.

Acetylcholine solutions were applied in 29 experiments. Small amounts (0.1—10 µg per ml) decreased the smell threshold in 15 of 20 experiments by 21—50 per cent corresponding to an air volume of 0.5 to 3.37 ml. A smaller or no decrease was produced in 5 experiments. In eight instances the threshold decrease was preceded by a transient increase in threshold of up to 27 per cent. There was no correlation between the concentration applied and the change in threshold (fig. 1).

Large amounts of acetylcholine (1 mg per ml) increased the smell threshold by 25—110 per cent in 8 of 9 experiments. This increase was not significantly different from that caused by the solvent alone. The increase was followed by a return to the initial level in 6 experiments (fig. 1) and a decrease in threshold in two. The change in threshold lasted 5 to 60 minutes depending on the

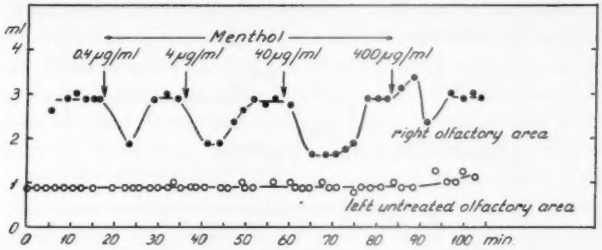


Fig. 2. Smell threshold variations of the right and left olfactory area of subject M. after application of menthol (0.4—400 µg per ml) to the right olfactory area at the times indicated by the arrows.

Ordinate: Smell threshold in ml air.
Abscissa: time in minutes.

amounts applied and the individual tested. No consistent change in threshold of the untreated olfactory area occurred.

Acetyl-beta-methylcholine in concentrations of 10 µg per ml produced decreases from the initial threshold value of 38—95 per cent.

Sodium chloride (0.9 per cent), the solvent used for both substances, produced increases of the smell threshold of up to 400 per cent in all of 22 experiments performed and never decreases (fig. 1).

Menthol solutions of 0.4—400 µg per ml were used in 26 experiments. The smallest amounts (<4 µg/ml) always decreased the threshold by 26—67 per cent. Larger amounts ($>4<400$ µg/ml) increased the threshold by up to 100 per cent in 6 of 9 experiments while decreases of 21—35 per cent were caused in 5. Solutions of over 400 µg/ml increased the threshold by 23—200 per cent in 6 of 7 experiments, while decreases were found in only three of these experiments and never exceeding 17 per cent. The threshold increases preceded always the decreases when both effects were produced in the same experiment (fig. 2). While the threshold decreases produced by small amounts are significant, the threshold increases with large amounts do not differ from those produced by the solvent. The effect on the threshold lasted five to sixty minutes. Large amounts of menthol produced in several experiments a transient prickling sensation and smell of menthol. This never occurred with solutions of 0.4—4 µg per ml. The contralateral smell threshold showed only insignificant variations.

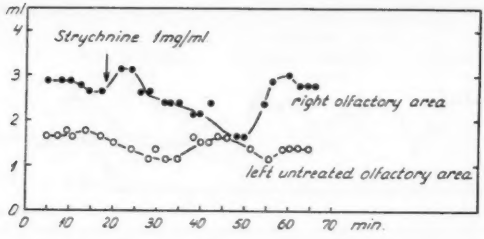


Fig. 3. Smell threshold variations of the right and left olfactory area of subject R. before and after the application of strychnine (1 mg/ml) to the left olfactory area at the time indicated by the arrow.

Ordinate: smell threshold in ml air.
Abscissa: time in minutes.

Strychnine nitrate in concentrations of 1 to 10 mg per ml was applied in 20 experiments. In all instances the threshold was first increased, with the weak concentrations (1 mg/ml) by 5 to 20 per cent; with stronger solutions by up to 900 per cent. This effect lasted approximately 10 minutes and was followed by a decrease in threshold of 15—40 per cent over about 30 minutes (fig. 3). Solutions containing less than 1 mg per ml did not differ from the solvent in their action on the threshold. Strong concentrations often caused a prickling sensation and bitter taste. The threshold level of the untreated area did not show consistent changes.

Discussion.

The experiments demonstrate that acetylcholine and acetyl-beta-methylcholine in suitable amounts produce a significant decrease of the smell threshold as determined by ELSBERG's method. This corresponds to previous findings on cold and pain receptors of the human skin, on thermo-receptors of the cat's tongue and on baroceptors of the carotid sinus of the cat. The long duration of the change in smell threshold found in the present experiments corresponds to the long lasting effect on thermo- and baroceptors in previous studies. This long lasting effect is remarkable in view of the known rapid destruction of acetylcholine.

The threshold decreases may be due to an action on the receptor cells directly or indirectly by influencing the nutrition of the receptive field or by facilitating the penetration of the stimulus to the receptor cell. It has been shown that in the skin acetylchol-

ine must act directly on the receptor cells or the terminal nerve fibres. Vascular effects were absent or not related to the sensitizing action. Neither acetylcholine nor menthol influenced the discharge from thermal receptors when applied to the afferent nerve fibres as close as possible to their endings (DODT, SKOUBY and ZOTTERMAN 1953). Therefore, assuming similar conditions in the nose, the effect of acetylcholine-like substances is most likely a direct one on the receptor. A similar mechanism may be assumed for menthol which also caused a decrease in smell threshold in small concentrations.

The strychnine experiments confirm the observation of FRÖHLICH 100 years ago that this substance increases the sensitivity to smell stimuli by a peripheral action. Strychnine influences ganglion cells, peripheral synapses and nerve fibres. Experiments with isolated preparations have shown that concentrations of less than $1:10^6$ exert an excitatory effect on ganglion cells while larger concentrations inhibit the response to electrical stimuli. The nerve axon was affected oppositely or not at all (HEINBECKER and BARTLEY 1939). The threshold decreasing effect demonstrated in the present experiments may, therefore, be produced by an action on the ganglion cells. Confirmation is found in that strychnine has not been demonstrated to produce a sensitizing effect on receptive areas without ganglion cells (FILEHNE 1901).

All the substances tested produced, in high concentrations, increases in threshold. In the human skin and the cat's tongue and the cat's carotid sinus, high concentrations of acetylcholine-like substances have a true depressant effect. In the present experiments, however, the increase in threshold with high concentrations cannot be attributed to depression, since the saline solvent alone raised the threshold in a similar way.

The occasionally found changes in smell threshold of the untreated area are probably due to spread of the solutions to the other side with the assumption of an upright posture.

Summary.

The smell threshold was examined by the method of Elsberg before and after the unilateral application of 0.9 per cent saline solutions containing small amounts of acetylcholine, acetyl-beta-methylcholine, menthol and strychnine. The threshold changes

observed were ipsilateral. Control experiments demonstrated that the solvent alone produced consistent increases in the smell threshold of up to 400 per cent.

Acetylcholine hydrochloride and *acetyl-beta-methylcholine* hydrochloride (0.1—10 μg/ml) decreased the smell threshold on the treated side by 21—50 per cent, as did *menthol* (4 μg/ml). *Strychnine* nitrate (1—10 mg/ml) decreased the threshold in all experiments, but after an initial increase in threshold lasting about 10 minutes. Higher concentrations of all the substances tested were associated with material threshold increases which did not, however, differ significantly from the threshold increase caused by the solvent alone.

The work was supported by grants from Frk. P. A. Brandts foundation and the P. Carl Petersen foundation.

References.

Bing, H. I. and A. P. Skouby, Acta Physiol. Scand. 1950. *21.* 286.
Dodt, E., A. P. Skouby and Y. Zotterman, Ibidem. 1953. *28.* 101.
Elsberg, C. A. and J. Levy, Bull. Neurol. Inst. N. Y. 1935. *4.* 5.
Filehne, W., Pflüg. Arch. ges. Physiol. 1901. *83.* 369.
Fröhlich, R., Sitzungsber. k. k. Akad. Wissensch. Wien. Math. nat. wiss. Klasse. 1851. *6.* 322.
Heinbecker, P. and S. H. Bartley, Amer. J. Physiol. 1939. *125.* 172.
Hensel, H. and Y. Zotterman, Acta Physiol. Scand. 1951. *24.* 27.
Landgren, S., A. P. Skouby and Y. Zotterman, Ibidem. 1953. *29.* 381.
Skouby, A. P., Ibidem. 1951. *24.* 174.
— Ibidem. 1953. *29.* 340.

From the Department of Pharmacology, Karolinska institutet, Stockholm, Sweden.

Photoelectric Recording of the Venous and Arterial Blood Flow.

By

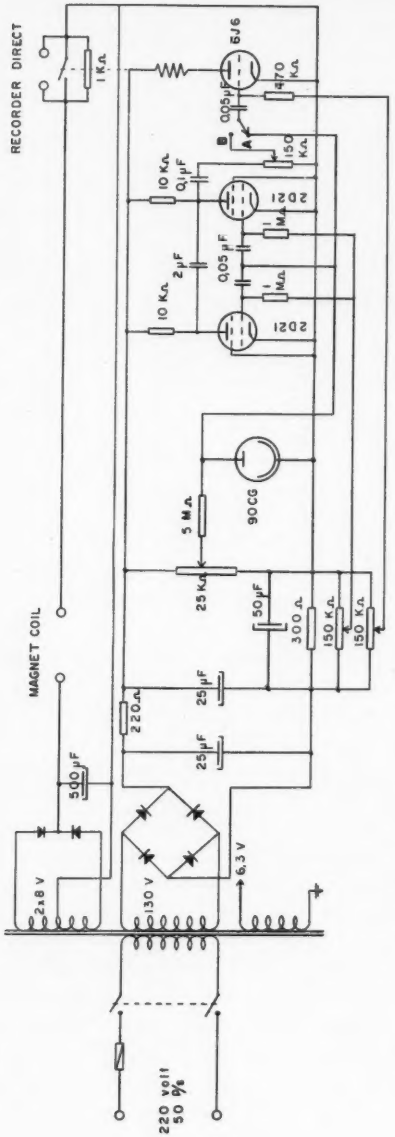
P. LINDGREN and P. UVNÄS.

Received 29 May 1954.

In the course of investigations on various circulatory problems since 1949, we have developed a method for recording the blood flow. As our experience with this method has been favourable, we wish to present it and to discuss certain of its details.

In principle, the outflow from a cannulated vessel is allowed to pass a photoelectric drop counter operating an ordinate writer. The original apparatus was described in detail by CLEMENTZ and RYBERG (1949). We shall therefore restrict the present discussion to the modifications of the apparatus made after the publication of their paper.

The frequency range of our original ordinate writer was from 5—250 impulses per minute. The range has later been increased to 2—500 impulses per minute. This improvement has been achieved by augmenting the transmission gear to eight gear steps. Furthermore, in order to avoid overworking the ordinate recorder at high frequencies, the electronic equipment has been rearranged so that, at will, either every or every second incoming impulse can be recorded. The figure shows the wiring diagram of the electronic equipment. It is seen that each impulse from the photocell reaches two separate circuits. If the switch is in position A, the impulses pass directly to the output tube (6 J 6) operating the magnet coil. If the switch is in position B, the impulses are relayed to the



output tube via a scale-of-two circuit; only every second impulse reaches this tube.

In its original version, the method could be used only for recording the venous blood flow. The anaesthetized animal was heparinized, a vein cannulated and the blood directed through a plastic tube to the phototube counter, placed on the same horizontal level as the animal. The blood was then collected in a glass vessel and returned to the animal by means of an intravenous drip infusion, the drop rate being continuously checked and adjusted to the magnitude of the outflow. In order to avoid this tedious manual control, the method was changed to a closed one. The venous outflow is now directed to a small drop chamber made of glass (Figure 2). The capacity of the chamber is 4—5 ml, and the drops fall from a height of 1—1.5 cm. The glass chamber has been mounted in such a way that it

Fig. 1. Wiring diagram of the electronic equipment in the ordinate recorder.

can easily be removed, opened and cleaned. From the drop chamber, the blood returns into the proximal end of the same vein or to some other vein, usually to an external jugular.

The change-over to a closed method has had several advantages. It is easier to observe simultaneously the blood flow in two or three different vascular areas. Moreover, the arterial blood flow can be recorded, at any rate in animals with a low pulse pressure.

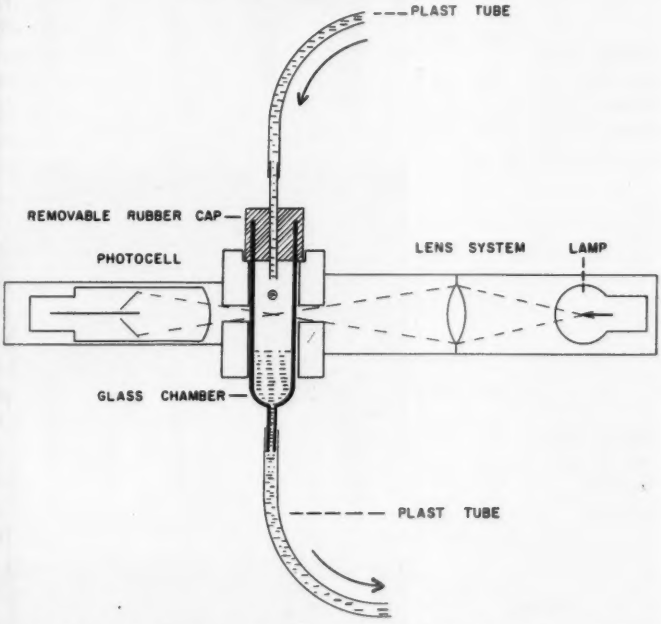


Fig. 2. Schematic drawing of the drop-chamber and the photocell arrangement.

Figure 3 is presented in order to show the nature of the blood-flow curves obtained with the method. In this experiment, the blood flow was recorded concomitantly in the femoral artery and vein in the skinned hind leg of a cat. To give some idea of the capacity of the method, it may be mentioned that vasodilator responses were elicited by intra-arterial injections of acetylcholine, 0.001—0.1 γ in 0.2 ml of physiological saline. The intra-arterial injections were made into the femoral artery, distal to the drop counter. As seen, the sensitivity to acetylcholine remained high.

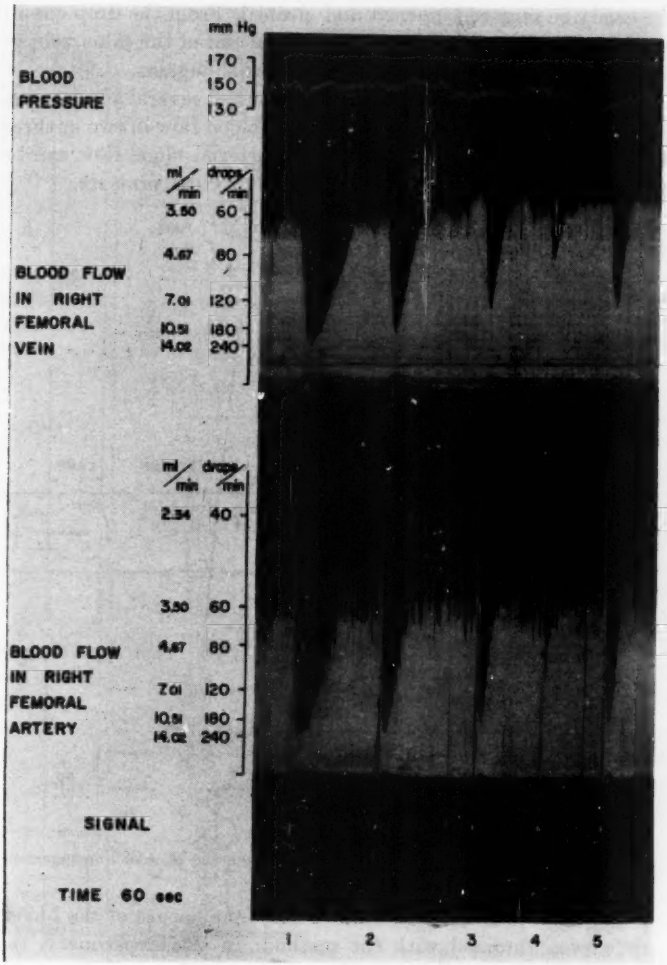


Fig. 3. Cat 4.0 kg.

Simultaneous recording of the arterial and venous blood flow. Vasodilator responses to intra-arterial injections of acetylcholine.

1) 0.1 μg of acetylcholine
2) 0.01 μg of acetylcholine
3) 0.001 μg of acetylcholine
4) 0.9 % NaCl
5) 0.001 μg of acetylcholine.

Pronounced vasodilator effects are produced by as small amounts as 0.001 γ. In the recording of the arterial blood flow, there are small irregularities. They are due to the influence of the pulse pressure on the arterial drop flow. This indicates that the method is suitable for recording the arterial blood flow only in animals with a fairly rapid heart rate (*e. g.* the cat and rabbit). Each intra-arterial injection causes a transient initial decrease in the arterial blood flow. This is due simply to the mechanical effect of the injection on the inflow to the drop chamber.

The volume of the extracorporal circulation in a drop chamber and its tube connections amounts to 5—6 ml. The inner diameter of the plastic tubes is 2 mm, and their total length about 45 cm. Under such conditions, the flow resistance in the recording unit is low. At a flow rate of 0.5—10 ml/min., it amounts to 1.5—4.0 cm H_2O.

The method is best suited for recording blood-flow rates from 0.5—20 ml/min. (corresponding to 10—350 drops/min.). This means, for example, a blood flow of a magnitude corresponding to the femoral flow in the cat and medium-sized dog.

The expenses of this investigation were partly defrayed by a grant from the Swedish Medical Research Council to one of us (B. U.) which is gratefully acknowledged.

References.

Clementz, B., and C.-E. Ryberg, Acta Physiol. Scand. 1949. *17*. 339.

Hilton, S. M., and D. W. Lywood, J. Physiol. 1954. *123*. 64 P.

From the Institute of Physiology, University of Upsala, Sweden.

The Permeability of Frog Skin to Urea with Special Reference to the Effect of Aminophylline.

By

LARS GARBY and HÅKAN LINDERHOLM.

Received 29 May 1954.

Isolated frog skin is known to transport actively sodium ions (LEVI and USSING 1949) and sometimes chloride ions (KOEFOED-JOHNSEN, USSING and ZERAHN 1952). Water also is transported actively inwards (HUF 1935), possibly as a result of the active sodium transport (USSING 1953). At the same time the skin probably behaves as a passive membrane to the particles mentioned as well as to most other substances (cf. LINDERHOLM 1954). Information concerning the resistance of the skin to various diffusing particles would be useful for a better understanding of the properties of the skin. The permeability of the isolated skin has been extensively investigated, but there have been few experiments that have allowed of a quantitative comparison of the permeability of the skin to particles of various known physico-chemical properties.

The flux of chloride ions (LINDERHOLM 1952) and heavy water (GARBY and LINDERHOLM 1953) through the isolated skin was studied. Simultaneously the direct current (d. c.) conductance of the skin was also recorded as a measure of the overall electrolyte permeability, thus making possible a comparison between different skins. The electrical potential across the skin was also recorded and was used as a criterion that the normal vitality of the skin was maintained during the experiment.

The results obtained invited speculation on the nature of the permeation process. If frog skin behaves as a "lipoid-sieve" mem-

brane (COLLANDER and BÄRLUND 1933) the permeation rate of lipoid insoluble substances should be mainly determined by the particle size. To find out if this is so, it would be of advantage to investigate the permeability to substances of different particle sizes. The number of suitable substances is limited. In this investigation urea was used for comparison with previous experiments. At the same time the effect of aminophylline on urea permeability was studied. This drug is known to have a pronounced effect on the permeability to chloride but little or no effect on that to heavy water (GARBY and LINDERHOLM 1953).

Methods.

Animals. The isolated abdominal skin of the green frog, *Rana temporaria*, was used. All experiments were performed in July. The animals were collected during the autumn and kept in a cold cellar in water tanks. Except for the last month they were fed with raw liver.

The general experimental arrangement was the same as that used by LINDERHOLM (1952) and GARBY and LINDERHOLM (1953). In short, the skin separated two compartments in a Perspex chamber unit that contained the bathing solution through which air was allowed to bubble. In the chamber electrodes were mounted for the measurements of the electrical potential and d. c. conductance across the skin. Ringer's solution was used in both compartments and was made up as follows: 9 parts of a solution containing 111.15 mM NaCl, 3.60 mM KCl and 3.81 mM/2 $CaCl_2$ and 1 part of a 100 mM phosphate buffer containing Na_2HPO_4 and NaH_2PO_4 in the proportions 16.16/1.92. The ionic strength was 0.135 and it contained 119.0 mM Na and 105.7 mM Cl per litre. The pH was 7.4—7.5. The temperature of the bathing solutions was kept at 20°.0 $\pm$ 0.1° C.

Performance of the experiments. The skin was mounted in the chamber as soon as possible after the frog had been killed. It was left for about 1—2 hours to stabilize. The bathing solution on one side was then replaced by a similar solution with a urea concentration of about 10 mM/l (Baker's urea pro analysi). Samples were subsequently drawn from both compartments at intervals of about 100 minutes, the first interval, however, being only 15—20 minutes. In general, immediately after removal of a sample, an aliquot volume of Ringer's solution (without urea) was added to the side, originally containing no urea. The volume of the compartments were recorded before and after sample removal and the potential and conductance across the skin was measured about every tenth minute.

Determination of urea concentration. The samples drawn from the compartments were transferred to test tubes, diluted, if necessary, to 0.4 ml, and incubated for 15 minutes at 37° C with 0.1 ml of urease

solution.[1] They were then immediately diluted to 15 ml and two drops of 1 % Gum Ghatti solution were added. Finally 1 ml of Nessler's reagent was added and after 5 minutes extinction values at 4,200 Å were read in a Coleman Junior Spectrophotometer. Blanks and standards were run in parallel with the samples.

Spontaneous urea production. Control experiments showed that appreciable amounts of urea were formed by the skin. The experiments lasted for 5—6 hours and samples were taken every 100th minute. The amount of urea produced during the various periods was determined. In three experiments without aminophylline the mean amount of urea given off to the outside solution was 0.017 μmoles/hr/cm^2 skin (range 0.01—0.047) and to the inside 0.034 μmoles/hr/cm^2 skin (range 0.003—0.064). In two experiments with aminophylline in the solution on the inside (50 mg/100 ml) the mean amount of urea given off to the outside was 0.017 μmoles/hr/cm^2 skin (range 0.003—0.029) and to the inside solution 0.050 μmoles/hr/cm^2 skin (range 0.047—0.067). The ranges include the values for each period.

This spontaneous urea production might be a result of the metabolic activity of the skin. In utilizing protein, an organism consumes 5.94l of O_2 for each gram of N or for 4.29 gram of urea excreted (DuBois 1927). The mean O_2 consumption of frog skin in Ringer's solution is 4.8 μlitres O_2/hr/cm^2 skin, and with aminophylline (50 mg/100 ml) about 50 % higher (Linderholm 1952, p. 126). If all the oxygen consumed was used in the combustion of protein a total amount of 0.058 (and with aminophylline about 0.09) μmoles of urea/hr/cm^2 skin should be formed. Thus the urea found in these experiments could be accounted for by protein breakdown.

In none of the experiments was ammonia detected. The same method as used for the urea determination was applied except that urease was not added and the incubation at 37° omitted.

Determination of the permeability coefficient. The permeability coefficient was calculated using Fick's law, which was integrated with the assumption that a linear concentration gradient existed through the skin. Assuming furthermore that the concentrations in the bathing solution and immediately under the membrane surface are equal, it follows that

$$k^{Ur} = \frac{v_1}{A \cdot \Delta t} \ln \frac{c_2 - c_1{}^{t_n}}{c_1 - c_1{}^{t_{n+1}}} \qquad (1)$$

when the urea was added to the inside solution (compartment 2). If urea was added to the outside solution (compartment 1) subscripts 1 and 2 change places. In eqn. 1 k^{Ur} is the permeability coefficient of urea (cm/sec.), v_1 is the volume (about 8 ml) of the outside solution in ml, A is the area of the skin in cm^2 (= 7.1 cm^2), Δt is the time interval

[1] Urease solution was prepared as follows: 0.1 g of urease powder (Coleman and Bell Co, Norwood, O., U. S. A.) was solved in 10 ml of phosphate buffer, equal parts of M/15 primary potassium and secondary sodium phosphate solution. It was shaken violently for 5 minutes and centrifuged. The supernatant was kept in a refrigerator at 0.5° C.

$t_{n+1} - t_n$ in seconds, c_2 is the concentration in compartment 2 (assumed to remain constant during the period, the mean value for the period being taken). $c_1{}^{t_n}$ and $c_1{}^{t_{n+1}}$ is the concentration of urea in compartment 1 (the superscripts indicating the beginning and end of the period), obtained by correcting the measured value for spontaneous urea production and volume changes when samples were drawn. The correction for spontaneous urea production was made by using the mean values given above.

The calculation of the permeability coefficient is thus liable to considerable error. In the first place the corrections for the spontaneous urea production are made by using mean values from controls on other skins. The scatter around these means is considerable, see above, and c_1 in eqn. 1 is then rather uncertain. Furthermore, the addition of urea made in the actual experiments may perhaps have changed the conditions for spontaneous urea production. These errors are much greater than those in the determination of the urea concentration.

Results.

The results are given in Table 1. The mean value for k^{Ur} of four skins without aminophylline is 0.64×10^{-6} cm sec.$^{-1}$ (range of the means for each experiment $0.38 - 1.01 \times 10^{-6}$ cm sec.$^{-1}$) and for four skins treated with aminophylline (50 mg/100 ml) 1.15×10^{-6} cm sec.$^{-1}$ (range of the means for each experiment

Table 1.

The urea permeability coefficient of frog skin, k^{Ur}, during the time intervals, Δt, is shown with the corresponding values of d. c. conductance, G. 2—1 denotes experiments where urea was added to the inside solution, and 1—2 where urea was added to the outside solution.

Skins without aminophylline				Skins with aminophylline (50 mg/100/ml			
Experiment no.	Δt min.	$k^{Ur} \times 10^6$ cm/sec.	G 1/kΩcm²	Experiment no.	Δt min.	$k^{Ur} \times 10^6$ cm/sec.	G 1/kΩcm²
1 (2—1)	120	1.93	0.87	5 (2—1)	119	0.25	3.15
	125	0.03	0.52		113	1.16	2.65
	121	0.19	0.43		115	1.00	2.09
2 (2—1)	139	0.18	1.02	6 (2—1)	93	0.83	1.72
	117	0.51	0.68		127	1.05	2.16
	104	0.62	0.50		104	2.40	1.84
3 (2—1)	106	1.87	1.45	7 (2—1)	101	0.75	3.42
	123	0.26	1.25		113	1.49	3.52
	117	0.90	0.97		124	2.23	2.60
4 (1—2)	112	0.14	1.12	8 (1—2)	109	0.10	2.50
	114	0.45	0.93		123	1.35	2.43
	109	0.54	0.82				
Mean		0.64	0.89			1.11	2.56

$0.73 - 1.49 \times 10^{-6}$ cm sec.$^{-1}$). In most of the experiments urea was added to the inside solution. In one experiment of each type it was added to the other side without apparently affecting the result. The difference between the means of the k^{Ur} of the two series, with, and without aminophylline, is not statistically significant. This is probably due to the small number of experiments performed and the possible errors discussed previously.

The means of the d. c. conductance, G, of the skins with and without aminophylline are 2.56 and 0.89 kΩ^{-1} cm^{-2} respectively and conform roughly with the values obtained earlier on a greater number of skins (LINDERHOLM 1952, GARBY and LINDERHOLM 1953). The electrical potential across the skin showed values between 10 and 40 mV, and also with respect to aminophylline the skins behaved as in earlier experiments. The present measurements on urea permeation may thus be compared with previous results on the permeation of other substances.

Discussion.

The methods used do not give very accurate values for the permeability coefficient of frog skin to urea. It is, however, assumed that the values obtained are correct enough to afford a basis for the discussion that follows.

It is interesting to compare the results with those obtained by GARBY and LINDERHOLM (1953). The permeability coefficient of frog skin to heavy water was found to be 73×10^{-6} cm sec.$^{-1}$ with a corresponding d. c. conductance, G, of 1.1 kΩ^{-1} cm^{-2}. For Cl ions a corresponding permeability coefficient was estimated to 1.5×10^{-6} cm sec. ($G = 1.0$ kΩ^{-1} cm^{-2}), and the present experiments with urea give a value of 0.64×10^{-6} cm sec.$^{-1}$ ($G = 0.9$ kΩ^{-1} cm^{-2}).[1]

The substances mentioned are practically insoluble in lipoids. The partition coefficients ether : water, olive-oil : water and olive-oil + oleic acid : water for urea (COLLANDER and BÄRLUND 1933) are respectively 0.00047, 0.00015 and 0.0052. They are therefore, in agreement with the lipoid-sieve theory, assumed to diffuse through the skin in water filled channels. At 25° C the diffusion coefficient for heavy water is 2.5×10^{-5} cm^2 sec.$^{-1}$ (WANG 1953),

[1] As there seems to be a slight positive correlation between the permeability coefficient of urea and chloride on one hand and the G-values on the other, it is probable that the permeability coefficient for urea is slightly too low to be compared with that of Cl ions.

that of Cl ions 1.88×10^{-5} cm² sec.$^{-1}$ (NIELSEN, ADAMSON and COBBLE 1952) and that of urea 1.39×10^{-5} cm² sec.$^{-1}$ (GOSTING and AKELEY 1952). The order of decreasing magnitude of the permeability and diffusion coefficients seems to be similar and compatible with the "pore" theory.

The permeation rate of water is much larger than that of Cl- and urea, and, cannot be accounted for by the difference in "free" diffusion rates. One is therefore tempted to conclude that water passes through the skin by pores that are not accessible to the larger particles. Conversely, it might be assumed that the three substances pass through the skin by way of similar pores. Then the relative and considerable restriction imposed on the Cl ions and the urea molecules must be explained and seems compatible only with the assumption that the available pores are of very small dimensions, indeed of similar size to the penetrating molecules.

In this connection it should be pointed out that several facts suggest that frog skin does not carry "fixed charges" (cf. TEORELL 1951) of any significance as regards ionic diffusion (LINDERHOLM 1952 and 1954), especially in the high electrolyte concentration used. Thus the size of the hydrated ion and not the charge should be the main factor that restricts permeation.

Aminophylline increases the permeability to Cl ions and probably to urea, but did not materially affect the water permeation (cf. GARBY and LINDERHOLM 1953). With regard to the experimental results it seems necessary to assume a very small increase in the effective diameter of the pores if the paths are common for the three particles mentioned, cf. the discussion in the earlier paper (GARBY and LINDERHOLM 1953). In case urea and Cl ions pass through some larger pores, essentially these would be affected by the aminophylline. Speculations as to the mechanism of this effect of aminophylline are probably futile at the present.

As is evident from this discussion the results give little possibility of forming a definite concept of the "pore structure" of the skin. Alternative explanations may be offered and more experiments with molecules of various properties are necessary for a more complete view.

Summary.

1. The permeability of isolated frog skins to urea was measured with a technique that allowed simultaneous measurements of

urea flux and of the d. c. conductance across the skin, *i. e.* the overall electrolyte permeability.

2. The permeability coefficient of urea was 0.64×10^{-6} cm sec^{-1}, a value about 100 times smaller than that for heavy water and about half the value of a corresponding coefficient for Cl ions.

3. Aminophylline seems to increase the permeation rate of urea at the same time as it increases the overall electrolyte permeability.

4. Possible mechanisms for the permeation of water, chloride and urea are discussed.

The investigation was aided by grants from the Swedish State Fund for Promotion of Medical Research and from the Therèse and Johan Andersson fund.

References.

COLLANDER, R. and H. BÄRLUND, Acta Botanica Fenn. 1933, *11*, 1.
DUBOIS, E. F., Basal Metabolism in Health and Disease, 2nd Edition, Philadelphia 1927.
GARBY, L. and H. LINDERHOLM, Acta Physiol. Scand. 1953, *28*, 336.
GOSTING, L. J. and D. F. AKELEY, J. Am. Chem. Soc. 1952, *74*, 2058.
HUF, E.: Arch. ges. Physiol. 1935, *235*, 655.
KOEFOED-JOHNSEN, V., H. USSING, and K. ZERAHN, Acta Physiol. Scand. 1952, *27*, 38.
LEVI, H. and H. USSING, Nature 1949, *164*, 928.
LINDERHOLM, H., Acta physiol. Scand. 1952, *27*, Suppl. 97.
LINDERHOLM, H., Acta Physiol. Scand. 1954, *31*, 36.
NIELSEN, J. M., A. W. ADAMSON and J. W. COBBLE, J. Am. Chem. Soc. 1952, *74*, 446.
TEORELL, T., Z. Elektrochem. 1951, *55*, 460.
USSING, H., in S. E. BRADLEY, *Renal Function*. Transactions on the fourth corference, 1952 p. 104, The Josiah Macy, Jr. Foundation, New York 1953.
WANG, J. H., C. V. ROBINSON and I. S. EDELMAN, J. Am. Chem. Soc. 1953, *75*, 466.

From the Department of Physiology, Karolinska Institutet, Stockholm.

A Frequency Compensated Input Unit for Recording with Microelectrodes.

By

L. HAAPANEN and D. OTTOSON.

Received 29 May 1954.

Introduction.

The capillary microelectrodes commonly used for measurements of bioelectric potentials exhibit certain electrical properties which lead to distortion of the recorded action potentials. The capillary microelectrode has been compared to a transmission line composed of several resistances and capacitances per unit length but an approximation is usually made and the equivalent circuit is represented by a single resistance and capacitance, the resistance being that of the electrolyte inside the electrode while the capacitance will be across the thin glass wall of the electrode (cf. *e. g.* WOODBURY 1952). Under recording conditions the shaft of the microelectrode is surrounded by extracellular fluid connected to ground and the capacitance mentioned above will thus be towards ground. A combination of a resistance and a capacitance is characterized by a time constant *i. e.* the product of the resistance in megohms and the capacitance in $\mu\mu$F (time constant in microsec.). When a step signal having an infinitely fast rise is applied to the tip of the microelectrode the time constant will cause a distortion by introducing an exponential delay of the rising phase. If *e. g.* the resistance of the microelectrode is 15 megohms and the capacitance 5 $\mu\mu$F the time constant will be 75 microsec., this being the time for the exponential rise to reach 63 % of the final

value. The time to reach full value will, however, be approximately 4 times the time constant *i. e.* 300 microsec.

Besides the time constant of the microelectrode proper certain stray capacitances exist in the input circuit of the recording amplifier and must also be taken into account when calculating the total time constant of the recording system. These stray capacitances are of two types: (i) the capacitance between the input grid and the other electrodes of the input tube, (ii) the capacitance between the input lead and ground.

In order to achieve minimum distortion of the recorded potential the total time constant must be kept as low as possible. When a signal is recorded through a high impedance device, such as a microelectrode, the cathode follower circuit has several advantages over the conventional amplifying stage (BISHOP 1949). One property of the cathode follower is to seemingly reduce any capacitance between grid and cathode. This feature has further been used in order to reduce the capacitance from grid input lead to ground by screening this lead and connecting the screen to cathode. In this manner the stray capacitances of the input circuit have been reduced to a low value. The total time constant will thus be determined mainly by the time constant of the microelectrode. As to results obtainable by use of the cathode follower NASTUK and HODGKIN (1950) in making intracellular recordings from muscle cells reported an over all time constant of 70 microsec. for an electrode of 22 megohms.

A radically different method of reducing the distortion when recording action potentials from single nerve fibers was used by WOODBURY (1952). The time constant of the recording system was of the order of 200—1,000 microsec. After amplification the distorted signal was electronically corrected in a mixer stage before display on the cathode ray oscillograph.

In connection with radio frequency transmitters a procedure known as neutralization has long been used in order to reduce the effect of grid to plate capacitance of a tube. An application of this method to low frequency amplifiers was made by ATTREE (1949) and more recently by KRAKAUER (1953) and HOGUE (1953). A separate amplifier was used to give positive feedback to the input circuit and cancel the effect of stray capacitances.

The principle of positive feedback has recently been applied by SOLMS, NASTUK and ALEXANDER (1953) in a preamplifier specially designed for recording with microelectrodes. Besides applying

positive feedback, two cascaded cathode followers were employed in order to further reduce the effect of stray capacitances. In measurements on a microelectrode of 9 megohms resistance an over all time constant of 7.8 microsec. was reported. In a paper on intracellular recording of action potentials of single muscle cells NASTUK (1953) concludes that a time constant of 30 microsec. or less is needed in order to get a faithful reproduction of the action potential. A design, apparently based upon the use of positive feedback, is also reported by WOODBURY (1953).

An input unit incorporating a frequency compensating circuit has been in use in this laboratory for some time. It has given similar results as regards reduction of the time constant to the design of SOLMS et al. The circuitry employed is rather simple, the frequency compensating effect being achieved by the use of two tubes only. For the input cathode follower a triode was chosen in preference to a pentode and the need for a separate screen supply battery was thus avoided. By applying positive feedback to the plate of this tube the effect of plate to grid capacitance was cancelled and the gain of the cathode follower made to approach unity.

Design of Input Unit.

The input unit, the circuit diagram of which is given in Fig. 1, was designed in order to achieve a device of great flexibility covering the demands of various types of experiments. In addition to the input cathode follower V_1 and the frequency compensating circuit requiring two tubes, V_2 and V_3, a conventional cathode follower V_4 was included in order to allow bipolar recordings.

The frequency compensating circuit is seen to consist of an amplifying stage V_2 the output of which is of the right phase to give positive feedback, and a second cathode follower V_3. The input tube, type 955, was chosen because of its small grid current. This tube, when operated under conditions given by BISHOP (1949), has a gain of approximately 0.8. It is desirable to increase the gain as far as possible towards unity in order to obtain a maximum reduction of capacitance between grid and cathode. This has been done by applying positive feedback to the plate of V_1, the plate voltage being furnished by the low impedance output of V_3. The degree of positive feedback applied to the plate of the

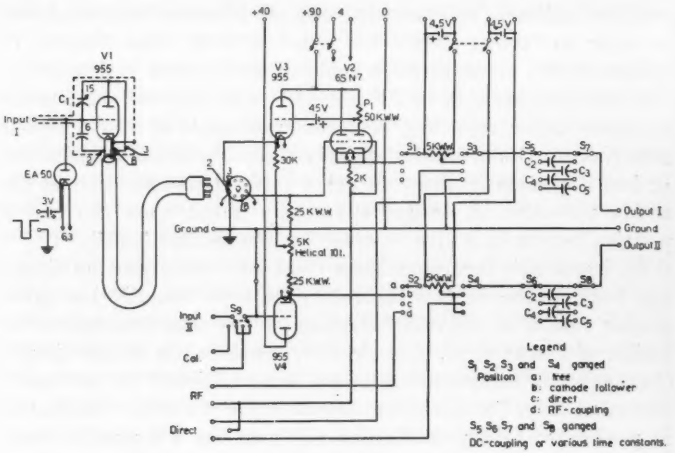


Fig. 1. Circuit diagram of the input unit.

input tube is controlled by a potentiometer P_1 in the plate circuit of the amplifying stage V_2.

The time constant of the microelectrode proper is reduced by neutralizing the capacitance to ground by applying positive feedback. This is performed by connecting a small variable capacitor C_1 between plate and grid of the input tube. This capacitor exhibits a zero capacitance of approximately 2 $\mu\mu$F which adds to the stray capacitance between plate and grid of the input tube and the total capacitance will thus be about 3.5 $\mu\mu$F. When the positive feedback control is set to give V_1 a gain close to unity this capacitance of 3.5 $\mu\mu$F will cause an overcompensation of the capacitance from grid to ground. To counteract the zero capacitance from plate to grid a fixed capacitor of the proper value is therefore connected between grid and ground. In our layout 6 $\mu\mu$F was found adequate.

The cathode of the input tube is at relatively high impedance level and therefore the output is taken across the low value cathode resistor of V_2.

Since recordings with a microelectrode are usually made against a large indifferent electrode of low resistance, frequency compensation was not applied to the cathode follower V_4. The input tube V_1 is enclosed in a lighttight metal container and placed close to

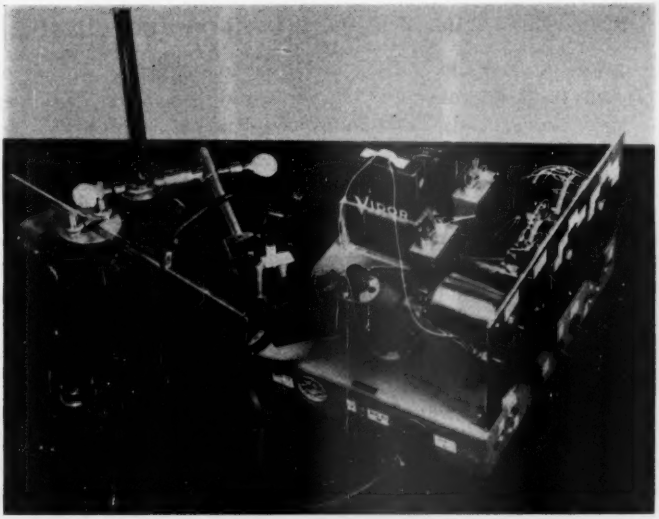


Fig. 2. View of input unit.

the preparation (see Fig. 2). The connection to the microelectrode is made short and is screened, the screen together with the metal container being connected to the cathode of V_1.

By means of a switch S_9 a calibration signal can be introduced between the indifferent electrode and ground. The input unit is provided with terminals by which connection can be made directly to the amplifier (switch position C in Fig. 1). Further a switch S_5—S_8 was included giving a choice of different low frequency response characteristics. Since the input unit is usually connected to a direct coupled amplifier (HAAPANEN 1953), the input signal of which must be at approximately ground level, two compensating circuits are provided. Terminals RF are used for connection of the special radio frequency coupled input stage developed in this laboratory (HAAPANEN 1952, HAAPANEN, HYDE and SKOGLUND 1953).

As to the layout of components and the wiring the usual precautions to reduce stray capacitances were taken. The condensers effecting the low frequency response were carefully matched and mounted on Perspex strips.

A general view of the input unit is shown in Fig. 2.

Properties of the Frequency Compensating Circuit.

Various methods have been suggested for the measurement of the time constant of microelectrodes and associated recording devices, under conditions simulating those of intracellular recordings.

NASTUK and HODGKIN (1950) measured the total time constant by applying a rectangular pulse to the tip of the microelectrode and observing the change in shape of the recorded pulse. Allowance was made for the influence of the capacitance across the glass wall of the microelectrode by mounting a drop of Ringer on the microelectrode close to the tip. The diameter of the drop was made to correspond to the depth of Ringer's fluid covering the preparation. The drop was connected to ground in order to imitate the conditions during recording. Another method was used by SOLMS, NASTUK and ALEXANDER (1953) who applied a square wave signal to the grid of the input tube through a high value resistor of 25 megohms. The microelectrode was then lowered into the Ringer's solution, to a depth corresponding to recording conditions and the compensating circuit was adjusted to give the closest approach to a square wave. Depending upon the characteristics of the 25 megohms resistor this method, however, may lead to an undercompensation because of the end to end capacitance of the resistor.

If a charge could be impressed on the grid-ground capacitance by means of a fast acting relay, this charge, upon release of the relay, would leak away through the resistance of the microelectrode at a rate corresponding to the time constant. The release time of relays, however, is too long and electronic circuits must therefore be employed.

In the present design this problem was solved by utilizing the characteristics of a vacuum tube diode. A diode can, depending on the value of plate cathode potential, be either conducting or non-conducting. The action is thus that of a relay and the charge to the grid circuit can be introduced through the diode.

The plate of a diode EA 50 is connected to the input grid of V_1 and the cathode of the diode kept at a positive potential of a few volts. The diode is then blocked but is made conducting for short time intervals by lowering the cathode potential in a square wave manner. Pulses with a distortion of the positive going phase, caused by the time constant of the grid circuit of V_1 will then be

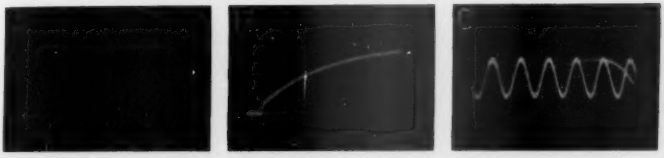


Fig. 3. Rising phase of rectangular pulse on cathode of EA 50 (A). Recorded pulse with R = 10 megohms and feedback controls at zero (B). Timing wave 10,000 c/s (C). See text.

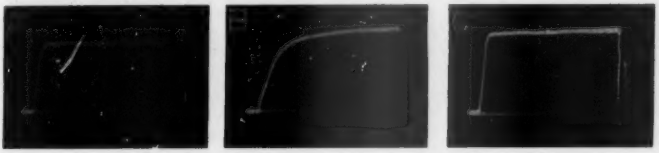


Fig. 4. Experiment on resistor. A, resistor only, critical setting of P_1. B, 3 $\mu\mu$F inserted from grid to ground. C, capacitor C_1 advanced to restore frequency response.

recorded. The grid current of V_1 was measured and found to be about $2 \cdot 10^{-11}$ A. No rise in this grid current could be observed when the diode was connected to the grid, heated, and the cathode of the diode kept at + 3 V.

In order to obtain a picture of the properties of the frequency compensating circuit in reducing the effective time constant a series of experiments was performed. First ohmic resistors and small fixed capacitors of different values were used to simulate the conditions when recordings with microelectrodes are made. The results obtained in this way were then compared with those of measurements on microelectrodes.

Measurements with resistors and capacitors replacing the microelectrode were made by connecting the input unit to an oscillograph provided with a condenser coupled wide band amplifier (2 c/s—2 Mc/s). Fig. 3 A shows the shape of the rising phase of the rectangular pulse applied to the cathode of EA 50. The rise time was below 1 microsec. as revealed by a faster sweep speed. When a resistor of 10 megohms is connected from grid to ground, the effective grid-ground capacitance of V_1 (about 13 $\mu\mu$F when the compensating controls are set at zero) will cause distortion of the rectangular pulse (Fig. 3 B). The time marking in Fig. 3 C is common to all the oscillograms in this and the following figures, the frequency of the sine wave being 10,000 c/s. In Fig. 4 A the

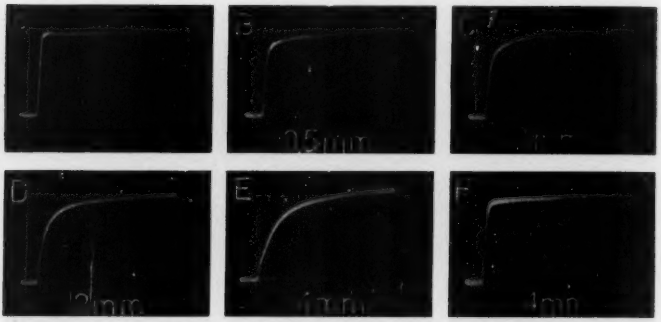


Fig. 5. Experiment on microelectrode of 15 megohms resistance. A, tip at surface, P_1 advanced and C_1 at zero. B—E, mm of insertion into grounded Ringer's solution indicated on records, P_1 and C_1 left unchanged. F, after correction with C_1.

resistor is again 10 megohms but the positive feedback control P_1 is adjusted for the best approximation to a square wave. The compensating capacitor C_1 was set at minimum. Fig. 4 B and C illustrate the effect of first increasing the capacitance from grid to ground by adding 3 $\mu\mu$F, and then neutralizing by increasing the value of C_1. In Fig. 4 C the time constant was measured as being approximately 16 microsec.

The microelectrodes used for control of the frequency compensating circuit were produced by an automatically operating pulling device of the type described by ALEXANDER and NASTUK (1953) and the electrolyte was 3 M KCl. Good uniformity of the electrodes was achieved by mounting the heater coil inside a glass tube in order to minimize the effect of eddy air currents which otherwise tend to make the temperature of the heater coil very critical. Resistance of the same order in microelectrodes drawn under similar conditions was taken as an indication that the differences in shape and tip diameter were also small. Our impression is, however, that no definite conclusion as to the tip diameter of a microelectrode can be drawn merely by using its electrical resistance as an index.

Fig. 5 represents a test carried out on a microelectrode of 15 megohms resistance as measured with a vacuum tube testmeter. In this experiment the microelectrode was placed with only the tip in contact with the Ringer's fluid connected to ground. P_1 was then advanced until the best response was obtained (A). B—E in the same Fig. show the gradual increase in time constant

Fig. 6. Experiment on microelectrode of 33 megohms resistance. A, tip at surface, P_1 advanced and C_1 at zero. B, tip at a depth of 4 mm. C, response after adjustment of C_1. Time constant about 20 microsec.

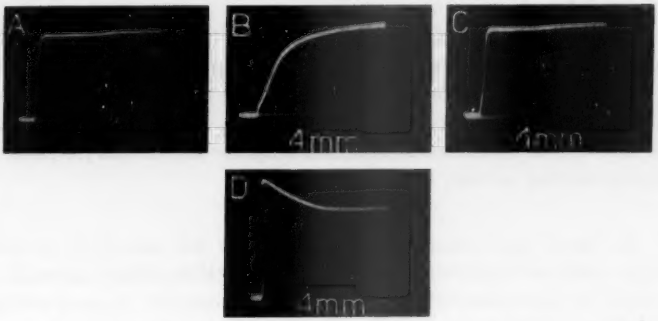


Fig. 7. Experiment on microelectrode of approximately 60 megohms resistance. A, tip at surface. B, tip at a depth of 4 mm. C, time constant after correction with C_1 about 14 microsec.

— due to the increase in capacitance — when the microelectrode was lowered into the Ringer's solution. In Fig. 5 F the neutralizing capacitor C_1 has been adjusted to reduce the time constant and restore the frequency response. In this case the time constant was about 14 microsec. Results from similar experiments on microelectrodes of 33 and 60 megohms resistance are presented in Figs. 6 and 7. Fig. 7 D shows the effect of overcompensation.

In order to prove that the procedure of adjusting the feedback controls P_1 and C_1 as described above, is valid also when recordings from an actual preparation are made, a means of imitating the cell membrane was sought for. Of the different types of artificial membranes investigated, a membrane produced from a commercially available plastic solution was found to be the most convenient. By blowing a balloon of a small amount of the solution an extremely thin membrane can easily be obtained.

Two pools of Ringer's solution were isolated from each other by such a plastic membrane, the upper pool being connected to ground and a variable frequency sine wave oscillator connected

to the lower pool. The microelectrode was advanced through the upper pool until the tip penetrated the membrane as revealed by the sudden appearance of the signal. The microelectrode passed through the membrane obviously on touching in a similar way as when advanced into a cell. The electrical resistance of the microelectrode was measured after the experiment and in almost every case found to be the same as before. This fact was taken as an indication that the tip of the electrode had not been broken or plugged by the membrane.

The frequency response, when tested in this way, was smooth and without increase in gain at certain frequencies, an effect which otherwise is easy to produce by advancing the feedback controls too far. Our conclusion therefore is that a faithful reproduction of fast action potentials is obtainable by the use of this frequency compensating circuit.

Summary.

An input unit incorporating a circuitry for reduction of the time constant when making recordings with capillary microelectrodes is described. Results of measurements on a number of microelectrodes with various resistances are presented. A considerable reduction in effective time constant under recording conditions was obtained. A method of adjusting the frequency compensating controls is given and a check of this method made by the introduction of a plastic membrane to simulate the membrane of a cell.

This work was supported by a grant from Svenska Sällskapet för Medicinsk Forskning.

References.

ALEXANDER, J. T. and W. L. NASTUK, Rev. Sci. Instr. 1953. *24*. 528.
ATTREE, V. H., Electronic Eng. 1949. *21*. 162.
BISHOP, P. O., Ibidem 1949. *21*. 469.
HAAPANEN, L., Rev. Sci. Instr. 1952. *23*. 337.
— Acta Physiol. Scand. 1953. *29*. Suppl. 106. 157.
— J. HYDE and C. R. SKOGLUND, Ibidem 1953. *29*. Suppl. 106. 394.
HOGUE, E. W., Electronics 1953. Nov. 184.
KRAKAUER, S., Rev. Sci. Instr. 1953. *24*. 496.
NASTUK, W. L., J. Cell. Comp. Physiol. 1953. *42*. 249.
— and A. L. HODGKIN, Ibidem 1950. *35*. 39.
SOLMS, S. J., W. L. NASTUK and J. T. ALEXANDER, Rev. Sci. Instr. 1953. *24*. 960.
WOODBURY, J. W., J. Cell. Comp. Physiol. 1952. *39*. 323.
— XIX Int. physiol. Congress 1953. 899.

From the Department of Medical Chemistry, University of Gothenburg, Gothenburg, Sweden.

Bile Lipids in Calves under Different Nutritional Conditions.

By

BJÖRN ISAKSSON.

Received 4 June 1954.

Human bladder bile contains a large amount of lecithin, constituting about 20 per cent of the solids (ISAKSSON 1951, POLONOVSKI and BOURRILLON 1952). The lecithin forms with part of the bile salts a system which is of importance for the dispersion of cholesterol in bile (ISAKSSON 1954). Thus it must be of interest to know conditions which can change the amount of lecithin in bile. Some biliary constituents have proved to be under the influence of diets. Such experiments however require relatively large experimental animals and are rather time-consuming and expensive. It therefore seemed wise to perform some preliminary analyses on bile from animals which were known to have had different nutritional conditions during their whole life. Calves were found to be very suitable for this purpose, as they are either given skim milk and then turned out to grass early in their life or reared on whole milk until being slaughtered. The results of an investigation on the biliary lipids from such animals, including newborn calves, are discussed in the present report. Determinations of the lipids in the serum and liver of the animals were performed for comparison.

Material.

The material is presented in Table 1. It was collected at the Public Slaughter-house of Gothenburg. The animals came from different farms.

Their ages were in some instances given by the farmer; otherwise they were assessed by the butchers and were therefore rather approximate. Unfortunately ages were not obtained during the first collection period (September 1953). It was not possible for the author to check the individual rearing. The forms of rearing, listed in Table 1, are in common use in this part of Sweden.

Table 1.

Average age and weight and the different forms of rearing in the experimental groups.

All animals were males of the S.R.B. breed (Swedish Red and White Cattle).

Group	Animals	No.	Age[1] average	Weight[2] average kg	Form of rearing	Collected
N	Newborn calves	5	< 1 w	27	Colostrum from the dam	September, 1953
W	Weal calves	10	7 w	55	Colostrum from the dam. Whole milk for 7—8 weeks. Then partly replaced by skim milk and concentrates	W 1—3, September, 1953, W 4—10, March, 1954
C	Calves (ordinary)	6	< 7 m	73	Colostrum from the dam. Whole milk for 2 weeks. Skim milk for 6—8 weeks. Then turned out to grass[3]	C 1—4, September, 1953, C 5—6, March, 1954

[1] The ages of the individuals are given in Table 2.
[2] The animals were weighed after the tripe had been taken out. The weights of the individuals are given in Table 2.
[3] Calves born in autumn (C 5 and C 6) or winter are usually given a fuller diet, including concentrates.

Methods.

The animals had not eaten the night before they were slaughtered. As soon as the animal had been killed the following samples were collected: blood was taken from the vena jugularis, the total contents of the gall bladder were squeezed out through the cut common duct, and a small portion of the liver was taken close to the gall bladder.

All the samples had been extracted or frozen within 5 hours of their collection. Extraction of the lyophilized biles was performed as described earlier (Isaksson 1951). The lipids in serum were extracted with ethanol/ethyl ether/acetone 1 : 1 : 1, in liver with ethanol/ethyl ether 3 : 1 after sectioning with freezing microtome following the method

of BRANTE 1949. After evaporation of the extraction solvents *in vacuo* the serum and liver lipids were taken up in chloroform and weighed.

Cholesterol was determined by the method of SCHOENHEIMER-SPERRY, modified by SPERRY and WEBB 1950. Total lipid-phosphorus was determined by FISKE-SUBBAROW's method as modified by BRANTE 1949, and the lecithin-choline according to the modification of BRANTE 1949. Mg P × 25 = mg phospholipids and mg choline × 6.4 = choline-lipids. Cholic acid in bile was determined by the method of IRVIN, JOHNSTON and KOPALA 1944.

The whole extraction procedure was performed once in every sample. All of the bile samples in groups W and C from the first collection period were controlled by a simplified extraction with ethanol only. The agreements were satisfactory. The analyses, except for part of the choline determinations, were performed in duplicate. The figures in the tables are mean values of all determinations. The accuracy of the methods will be reported in a subsequent investigation (ISAKSSON 1954) partly worked out during the same period as the present one.

Results.

Bile (Table 2). There were no differences in the general appearance of the biles; the colour was always faintly green, thus deviating from that of human bile. The amount of *total solids* was low in some N-calves, possibly owing to undeveloped concentration power of the gall bladder. The difference between groups W and C is not statistically significant.

All other components examined were expressed as a percentage of total solids, thus eliminating the influence of the concentration powers of the gall bladder. On the other hand, a high percentage of one of the constituents may be due to low amounts of the others. Determinations of cholic acid were therefore always performed. It was regarded as representative of the bile acids which constitute the largest part, about 60 %, of the solids. A low percentage of cholic acid might perhaps be regarded as a sign of low output of all bile acids from the liver. Thus in group N, the mean value for cholic acid is only about 2/3 of the mean value for the other groups. For comparison the mean figures for cholesterol and lecithin in group N should therefore possibly be reduced to about 0.8 and 12 per cent respectively.

Cholesterol. The extraction of cholesterol from lyophilized bile from cattle does not succeed with ethyl ether only, as has been pointed out earlier (ISAKSSON 1951). For quantitative purposes it is thus necessary to analyse the subsequent chloroform

Table 2.

Distribution of lipids (and cholic acid) in bladder bile from calves.

No.	Weight	Age	Total bile solids	Lecithin	Total—P / Lecithin-choline	Cholesterol	Cholic acid
	kg		g/litre	as a percentage of solids			
Group N (Newborn calves)							
N 1	30	< 1 w	33	19.4	0.94 : 1	—	16.2
N 2	23	»	81	23.7	—	1.6	14.1
N 3	28	»	47	16.0	1.06 : 1	—	24.9
N 4	25	»	65	14.6	0.97 : 1	0.9	21.3
N 5	28	»	65	16.7	1.18 : 1	1.1	25.8
Mean	27	< 1 w	58	18.1 (12.0)	1.04 : 1	1.2 (0.8)	20.5
Group W (Weal calves)							
W 1	49	—	70	15.5	—	0.9	31.2
W 2	64	—	90	15.5	1.07 : 1	1.3	27.4
W 3	84	—	94	16.5	0.99 : 1	1.2	25.1
W 4	60	8 w	103	19.1	1.10 : 1	1.7	21.9
W 5	54	10 w	78	19.6	0.95 : 1	1.0	25.3
W 6	48	7 w	67	16.0	1.07 : 1	1.0	22.2
W 7	43	4 w	69	15.4	0.99 : 1	1.2	27.3
W 8	46	7 w	91	16.5	0.91 : 1	1.2	28.0
W 9	56	9 w	77	15.9	0.80 : 1	0.8	34.2
W 10	50	7 w	88	15.4	1.08 : 1	0.7	30.6
Mean	55	7 w	83	16.6±0.5	1.00 : 1	1.1±0.09	27.3
Group C (Calves)							
C 1	51	—	99	9.9	1.11 : 1	0.6	32.3
C 2	83	—	87	10.8	0.99 : 1	0.6	29.4
C 3	75	—	92	7.5	—	0.8	40.4
C 4	63	—	86	6.5	—	—	34.0
C 5	86	7 m	150	11.7	0.95 : 1	0.7	23.8
C 6	78	4 m	78	12.3	0.91 : 1	0.8	30.3
Mean	73		99	9.8±0.9	0.99 : 1	0.7±0.04	31.7

and ethanol extracts. The figure in Table 2 represents the sum from all solvents. In some instances esterified cholesterol is actually found, and cholesterol esters possibly occurred in the other biles too. As regards total cholesterol the difference between groups W and C is statistically significant (0.4 ± 0.10).

Lecithin was demonstrated to be the only phospholipid in the calf bile, and the same was found in all species of animals previously examined by the author. The value of the ratio between the total phospholipids and KOH-splittable choline-lipids above

Table 3.

Lipids in the serum of the calves.

	Total lipids	Phospho-lipids	Lecithin	Cholesterol	
				Total	Bound
	in mg per 100 ml serum				as a per-centage of total
Group N (Newborn calves)					
Range	338—580	68—147	53—143	47—102	70—79
Mean	469± 59	110± 19	100± 25	72± 13	74± 2
Numbers	4	4	4	4	4
Group W (Weal calves)					
Range	250—610	53—189	56—162	51—124	64—86
Mean	433± 32	122± 12	105± 10	91± 7.7	75± 2.4
Numbers	10	10	10	10	9
Group C (Calves)					
Range	248—468	66—151	33—117	47— 66	47—68
Mean	381± 32	100± 12	65± 16	59± 3.5	61± 3.6
Numbers	6	6	5	5	5

the range 0.90—1.10 was possibly due to an erroneous determination. The difference between mean values of lecithin in groups W and C is highly significant (6.8 ± 1.1). The value for the "reduced" N-group is not significantly higher than the one in C but lower than the one in W.

Other lipids such as free fatty acids and neutral fat have been recognized in the ether extract only. The amounts are however so small, and the methods for their determination so inaccurate, that the figures are not given.

Serum (Table 3). A relatively wide range for all components is apparent in every group, which makes the average value uncertain, especially in the small groups N and W. Nevertheless a statistical evaluation shows significant differences between W and C concerning the cholesterol and percentage esterified cholesterol ($P < 0.01$) but not concerning total phospholipids and lecithin.

Liver (Table 4). The dispersion in each group is not so apparent for the lipids in the liver as for those in serum. A statistical evalua-

Table 4.

Lipids in the liver of the calves.

	Total lipids	Phospho-lipids	Lecithin	Cholesterol	
				Total	Bound
	as a percentage of dry substance				as a percentage of total
Group N (Newborn calves)					
Range	17.0—19.8	8.4—10.1	3.9—5.4	0.8—1.0	22—31
Mean	18.2± 0.5	8.9± 0.3	4.8±0.1	0.9±0.03	28± 2
Numbers	5	5	4	5	4
Group W (Weal calves)					
Range	19.3—20.9	8.8—11.5	3.5—5.7	0.7—1.0	12—26
Mean	20.3± 0.5	9.9± 0.8	4.3±0.7	0.8±0.10	20± 4
Numbers	3	3	3	3	3
Group C (Calves)					
Range	21.0—25.2	11.4—12.8	6.0—7.2	0.8—0.9	27—28
Mean	23.2± 0.9	12.2± 0.3	6.7±0.3	0.9±0.04	28
Numbers	4	4	4	4	2

tion is of little value because of the small number of analyses. From the figures, however, a larger amount of lecithin in group C compared with the other groups is unquestionable. The other lipids are of the same magnitude in all groups.

Discussion.

The first question is, are the differences found between the groups in the composition of the biles due to differences in age or in diet, or to both?

The figures for the mean age and for the mean of cholesterol or lecithin in each group might suggest a dependence on age. However, this is not borne out if each group is examined separately. Thus, the ages of 7 W-calves are known, but this material does not support the assumption that the cholesterol or lecithin level decreases with increasing age. Neither is this the case with the 2 C-calves with known ages. Moreover, although a positive correlation between age and weight can be assumed to be present within each group (otherwise the reason might be differences in rearing), the figures in Table 2 give no evidence of a correlation

between the weights and the amounts of any of the lipids investigated. Age, therefore, does not seem to have any marked influence on the output of lipids in the bile of groups W and C. The differences may thus in all probability be ascribed to the different forms of rearing between the groups.

Only in group N is it nevertheless possible that the age has greatly affected the composition of the bile. This possibility cannot in any case be excluded, and the results of the analyses are therefore only given but not discussed in the present report.

In the literature a lot of reports exist on the influence of diets on the composition of the bile. The results are, however, very conflicting, certainly because different methods of collection have been used. Analyses of duodenal, fistula or bladder bile taken before or after death can hardly give the same results, especially as the amounts have been expressed in concentration units. The species of animal might of course also have been of importance. For the lipids, in addition, appropriate methods have seldom been used (for discussion, see ISAKSSON 1951). Thus it seems to be of little value to review the earlier investigations in this field in any great detail.

Cholesterol. Our knowledge of the metabolism of cholesterol is not sufficient to explain the differences in the amount of biliary cholesterol between the groups in the present investigation. From the works of WHIPPLE (1922), DOSTAL and ANDREWS (1933), and BYERS and FRIEDMAN (1952) it seems, however, to be clear that there is no connection between dietary and biliary cholesterol. The recent report of BYERS and FRIEDMAN (1952) makes it more probable that the rate of synthesis of cholesterol in the liver determines the daily output of cholesterol in bile. In order to compare the cholesterol excretion activity of the liver of the animals investigated, the figures for the mean amount in mg/ml bile per kg body weight may be used: group N 0.025 ("reduced" N 0.017), W 0.017 and C 0.010. The synthesis activity of the liver for cholesterol has been found by several investigators to be under influence of different conditions. The influence of different nutritional conditions has been studied with conflicting results. In order to be able to make a contribution to the discussion, the present investigation ought to be repeated with complete control of the rearings of the calves. As the most striking difference in the composition of the diets between groups W and C lies in the fat content, an experimental series on weal calves with different

percentages of fat in the milk has been started, and the results will be reported elsewhere.

As the liver is also known as the primary source of the serum cholesterol in normal animals, the results in Table 3 for groups W and C favour the idea of differences in its synthesis activity for cholesterol. The esterification of cholesterol is not a function of the liver, but an enzyme in liver homogenisates which hydrolyses cholesterol acetate has recently been reported (SCHOTZ 1954). This enzyme is as yet very little studied and the significant difference in the percentage of ester cholesterol in serum between the groups is unexplained at present. The absence of differences between the groups in the amount of liver cholesterol seems to indicate that none of the present nutritional conditions enhances an accumulation of cholesterol in the liver.

Phospholipids. A survey of the recent literature gives only a few reports on investigations of the origin of the biliary lecithin. SCHAFFNER and co-workers (1951) measured the specific activity of biliary and plasma phospholipids after the injection of P^{32} into man and dog. The amount of the isotope incorporated in the biliary phospholipids was very small and did not show any correlation to the amount incorporated in plasma phospholipids. The lipid extraction procedure was not described, but it does not seem to have been satisfactory, as they report that "the total phospholipid in bile is low compared with plasma". As mentioned in the present introduction, the lecithin content is high in man (as it is also in dog), and the concentration in the gall bladder is about ten times that in serum. In liver bile it therefore might be of the same order as in serum. COLWELL (1951) studied the lipid content of bile secreted by choline-deficient rats with fatty liver. Again the method for the lipid extraction was not adequate, as he reported that the phospholipid content was less than 0.5 % of the total lipids. The amount of lecithin in rat bile is however of the same magnitude as in man. Thus the origin of the biliary lecithin is still unknown.

From the figures in Table 2, a calculation of the ratio between lecithin and cholesterol gives nearly the same mean values in all three groups: N 15.4, W 15.6 and C 15.2. The daily output of lecithin in mg/ml per kg body weight in the bile will thus give the same ratio between the groups, as was the case with cholesterol. For the present author it therefore does not seem unlikely that a mechanism, similar to the one for the biliary

cholesterol is responsible for the differences in lecithin level between the groups. It is also possible that there exists another form of connection between the outputs of cholesterol and lecithin. Again, it is seen to be necessary to perform experiments with controlled rearings of the calves.

Finally, it seems well to point out that the high daily output of lecithin in the intestines via the bile in some animals is a point that must be noted by investigators of the mechanism of fat absorption. For various purposes a phospholipid-free diet might be quite necessary. This does not mean, however, that the intestines are unsupplied with lecithin if bile is allowed to enter into them. This does not seem to have been noted before.

Summary.

The lipids in bladder bile collected from newborn calves, weal calves and ordinary calves were investigated. For comparison the same determinations were performed on their sera and livers.

It was found that the weal calves had a much higher percentage of cholesterol and lecithin in the bladder bile than the ordinary calves. The same was true when the daily output of both components was expressed in mg/ml per kg body weight.

The differences were attributed to differences in nutritional conditions. In the newborn calves the values for biliary cholesterol and lecithin were regarded as somewhat lower than in the weal calves and attributed to their age.

The importance was pointed out of due regard to the bile lecithin during *in vivo* studies of intestinal fat absorption.

This is part of an investigation partly supported by a grant from the Swedish State Fund for Promotion of Medical Research. The author is indebted to Mr. Tore Lundin at Schaub and Co, Gothenburg, for all the necessary arrangements at the Slaughterhouse and to the butchers for help with the collection of the samples. Thanks are due to Miss Ulla-Britt Josefsson and Mr. Åke Lundquist for valuable assistance.

References.

Brante, G., Acta Physiol Scand., 1949. *18*. Suppl. 63.
Byers, S. O., and M. Friedman, Amer. J. Physiol., 1952. *168*. 297.
Colwell, A. R., Ibidem, 1951. *164*. 274.

Dostal, L. E., and E. Andrews, Arch. Surg., 1933. *26*. 258.
Irvin, J. L., C. G. Johnston and J. Kopala, J. Biol. Chem.. 1944. *153*. 439.
Isaksson, B., Acta Soc. Med. Upsaliensis, 1951. *56*. 177.
— Ibidem 1954. *59*. 277, 296.
Polonovski, M., and R. Bourrillon, Bull. Soc. Chim. biol., Paris, 1952. *34*. 712.
Schaffner, F., M. Meitus, J. de la Huerga, D. F. Magee, F. Steigmann and H. Popper, Fed. Proc., 1951. *10*. 369.
Schotz, M. C., L. I. Rice and R. B. Alfin-Slater, J. Biol. Chem., 1954. *207*. 665.
Sperry, W. M., and M. Webb, Ibidem, 1950. *187*. 97.
Whipple, G. H., Physiol. Rev., 1922. *2*. 440.

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